Studying Pathogen-Host Interactions AND Pathogenesis IN *Cronobacter* AND *Listeria* Infections Using *In vitro* AND *In vivo* Infection Models

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PREFACE

“The only time success comes before work is in the dictionary.”

- Walter Bagehot -

SUMMARY

In order to elucidate mechanisms of pathogenesis of human bacterial pathogens the availability and application of suitable *in vitro* and *in vivo* models is indispensable. Within this thesis I was aiming to make an important contribution to answer open questions concerning the host-pathogen interactions as well as virulence factors and mechanisms for two opportunistic bacterial pathogens namely *Cronobacter (C.)* spp. *and Listeria (L.) monocytogenes.*

Cronobacter spp. can cause severe infections in the intestine and central nervous system predominantly in pre-term infants and neonates with high mortality rates. Several aspects of its pathogenesis such as interactions with the host immune system or the molecular mechanisms of bacterial uptake and distribution within the host still remain elusive.

L. monocytogenes is known to be the aetilogical agent of listeriosis, a disease that leads to serious illnesses and high mortality rates within the populations of risk, as well as to spontaneous abortions and still births. In contrast to *Cronobacter* spp. mechanisms of infections with *L. monocytogenes* have been studied quite thoroughly. However, the role of stress related proteins to promote virulence of these pathogens has not yet been deeply explored.

Two model systems – the *in vitro* phagocyte (macrophage) infection and the zebrafish embryo *in vivo* model were applied to study open questions concerning the pathogenesis of these pathogens. Macrophage uptake of invading microbes is an innate process, and the capability of pathogens to survive and/or replicate within macrophages resembles the first step to bypass the host immune system and to establish an infection.

The zebrafish (*Danio rerio*) as has previously been shown to be an appropriate *in vivo* model organism to study bacterial infection with (human) pathogens due to its small size, optical transparency, genetic manipulability and the physiological similarities with mammals.

By application of these two models we could demonstrate that *Cronobacter* harbor the *mip* (macrophage infectivity potentiator)-like gene *fkpA*, which promotes intracellular survival of

these bacteria in human macrophages and which was considered to date as unknown virulence factor. Moreover, the presence and function of a novel quorum sensing based regulatory system in *Cronobacter* spp. was elucidated and further explored using the *in vivo* model. In addition, the zebrafish embryo model was successfully applied to get insights into the varying virulence behavior among strains within the *Cronobacter* genus. Thereby, the usefulness of the zebrafish embryo model to study virulence factors on a "large scale" was demonstrated.

Furthermore, the role of the cold shock domain proteins (Csps) in *L. monocytogenes* and their contribution to virulence was studied in detail for the first time using these models.
In conclusion, we showed that both models represent valuable tools to study mechanisms of infections caused by the two pathogens *Cronobacter* spp and *L. monocytogenes.*

Zusammenfassung

Für die Aufklärung zur Entstehung bzw. des Verlaufs (Pathogenese) von humanen Erkrankungen, die von bakteriellen Erregern ausgelöst werden ist das Vorhandensein sowie die Anwendung geeigneter *in vitro* und *in vivo* Modelle von eminenter Bedeutung. Ziel meiner Arbeit war es, einen wichtigen Beitrag zur Aufklärung offener Fragen bezüglich Wits-Pathogen Interaktionen bzw. zu Virulenzfaktoren und –mechanismen für die beiden oppotunistisch pathogenen, bakteriellen Erreger *Cronobacter* (C.) spp. und *Listeria (L.) monocytogenes* zu leisten.

Cronobacter spp. können schwerwiegende Infektionen im Darm bzw. dem zentralen Nerversystem vor allem in Früh- und Neugeborenen verursachen, welche mit hohen Mortalitätsraten einhergehen. Verschiedene Aspekte der Pathogenese, wie z.B. die bakterielle Interaktion mit dem Wirts-Immunsystem oder die molekularen Mechanismen welche während der Aufnahme der Bakterien bzw. deren Verteilung innerhalb des Wirtsorganismus zum Tragen kommen sind noch nicht ausreichend erforscht.

Listeriose wird durch Infektion mit *L. monocytogenes* verursacht welche ebenfalls schwere Erkrankungen mit hohen Mortalitätsraten in Risikopopulationen hervorrufen kann. Infektionen mit *L. monocytogenes* können zudem zu spontanen Fötusabgängen und Totgeburten führen. Im Gegensatz zu *Cronobacter* spp. sind die Mechanismen, die zu Infektion und Krankheit führen bei *L. monocytogenes* schon um einiges besser erforscht.
Einige Aspekte jedoch, wie z.B. jener der Rolle die die Stressantwort Proteine bei der Virulenz spielen wurden bis dato nicht noch nicht eingehend untersucht.

Zwei verschiedene Modellsysteme – das *in vitro* Phagocyten (Makrophagen) Infektionsmodell und das *in vivo* Zebrafisch Embryo Modell wurden in dieser Studie angewandt um offen Fragen bezüglich der Pathogenese dieser beiden Erreger zu beantworten. Die Aufnahme von invadierenden Erregern durch Makrophagen ist Teil der angeborenen Immunabwehr und die Fähigkeit von pathogenen Erregern in diesen zu überleben oder sich darin zu vermehren ist der erste Schritt die Immunabwehr des Wirtes zu umgehen und eine Infektion zu etablieren.

Zusammenfassung

Der Zebrafisch (*Danio rerio*) hat sich in der Vergangenheit bereits vielfach als geeignetes *in vivo* Modell zur Erforschung der Pathogenese von (humanen) pathogenen Erregern erwiesen vor allem auf Grund seiner kleinen Grösse, optischen Transparenz, der Möglichkeit zur genetischen Manipulation und seiner physiologischen Ähnlichkeiten zu Säugetieren.

Die Anwendung dieser beiden Modelle ermöglichte uns die Charakterisierung eines Gens - des *mip* (macrophage infectivity pitentiator) – like Gens *fkpA in Cronobacter,* welches wesentlich zum Überleben in humanen Makrophagen beiträgt und daher als Virulenzfaktor bezeichet werden muss. Desweiteren wurde das Vorhandensein und die Funktion eines neuen, auf Zell-Zell Kommunikationssystem basierenden Regulationssystems in *Cronobacter turicensis* bestätigt und aufgeklärt und dessen Einfluss auf die Pathogenese durch Anwendung des *in vivo* Modells näher charakterisiert.

Darüber hinaus wurde das Zebrafisch Embryo Modell erfolgreich angewandt, um Antworten auf die Frage der beobachteten Varianz bezüglich der Virulenz bei Stämmen innerhalb des Genus *Cronobacter* zu beantworten. Dabei konnte auch demonstriert werden, dass das Zebrafisch Embryo Modell bestens geeignet ist, um die Rolle von Virulenzfaktoren im “Hochdurchsatz” aufzuklären.

Die Anwendung beider Modelle ermöglichte desweiteren die Rolle der “cold shock domain proteins” (Csps) in *L. monocytogenes* für die Virulenz zum ersten Mal im Detail zu studieren. Zusammenfassend konnten wir zeigen, dass beide Modelle einen wertvollen Beitrag leisten können, um Infektionsmechanismen bei diesen beiden Erregern - *Cronobacter* spp. und *L. monocytogene*s aufzuklären.

Chapter 1

General Introduction

1.1 Infection and Infectious diseases

An infection is invasion, colonization and multiplication of infectious agent inside an organism's body and causing harm to the host. Infectious agents can either be bacteria, viruses, fungi, viroids, nematodes and parasitic worms. Hosts can combat infections by using their immune system, mammalian hosts can fight infections with an innate immune response, often involving inflammation, tailed by adaptive immune response. Infectious diseases are one of the major causes of death among humans. Some infectious diseases can be acquired by ingesting food contaminated with foodborne pathogens making it a major issue in food production industry.

1.1.1 Opportunistic bacteria

Although most bacteria are harmless or often beneficial, few of them are pathogenic. The largest numbers of bacteria and the greatest number of species are present in human gut microbiota (Quigley 2013) and they play vital role in fermenting dietary fibers along with other important functions. There are about 10^9 different species of bacteria in the world (Schloss et al., 2004) but only about 8000 have been described to date of which 518 species are known to be pathogenic to humans (Cleaveland et al., 2001). Bacteria are known to be the oldest forms of life and have constantly evolved to utilize energy from different energy sources such as sunlight, inorganic compounds etc. For example, iron is required as a nutrient for humans as well as growth of most bacteria. Some bacterial species are fully dependent on a host cell for supply of energy and nutrients making them obligate intracellular pathogens. However facultative intracellular bacteria can survive and replicate both inside and outside of the host cell. These bacteria can be found in the environment invade host cells under certain circumstances or when it gives them selective advantage in the host such as immune compromised individuals or infants with premature immune system hence are called opportunistic pathogens. These invading bacteria must possess

specialized mechanisms to protect themselves from encounters of lysosomal enzymes within the host cell. For example, *Legionella pneumophila* encourages its own uptake and inhibits lysosomal fusion, *Mycobacterium tuberculosis* blocks phagosome-lysosome fusion and *Listeria monocytogenes* swiftly escapes into the cytoplasm from phagosome before the fusion of phagosome and lysosome.

1.2 Phagocyte model

Macrophage uptake of invading microbes is an innate process as part of the first line of defense towards eliminating harmful pathogens. The ability of some bacteria to persist and replicate within these immune cells is important for bacteria to cause an infection and a primary step in development of severe illnesses such as sepsis or meningitis. Bacterial strains unable to survive the intracellular environment may be considered avirulent. An *in vitro* phagocyte infection model provides an overview of the pathogen's ability to survive and replicate inside the cell however this model also has limitations wherein it does not provide the holistic view of the infection process, loss of function due to purification of macrophages, culture media does not summarize tissue specific in vivo nutrients, absence of extracellular matrix interactions and soluble factors such as opsonins and cytokines, absence of other cell types for contact activation and inhibition (Tobin et al., 2012). In order to overcome these limitations and to monitor dissemination of infection, development of animal models is essential.

1.3 Animal models of disease and its applications

Humans and animals may look different but are strikingly similar at physiological and anatomical level. In comparison to humans, animals have same organs and organ systems which perform functions nearly in the same way as humans. They are so similar that almost 90% of the veterinary medicines are same as or very similar to, those developed to treat humans. Even though there are dissimilarities, these are extremely overshadowed by the similarities. Model organisms can be used as host to pathogens to study host-pathogen interactions. This will provide better understanding of host immune responses to the infection and also tactics employed by pathogen to overcome and thrive within the host. The testing of potential antimicrobial agents can also be performed in animal models of

infectious diseases there by bridging the gap between *in vitro* characterization and the clinical assessment of antimicrobial agents (Zak et al., 1991). Preferred features of a model organism are that they should be easy to handle during experiments, possibility to rear them in large numbers in laboratory conditions, well explained structural, genetic and behavioral features and genetic manipulability. However, it is difficult to find a model organism that can satisfy all the above requirements. Even the most widely used animal models have limitations. For example, *Drosophila melanogaster* also known as fruit fly is popular for its shorter life cycle, inexpensive to culture and maintain, produce large numbers of externally laid embryos and they can be altered genetically in many ways (Jennings 2011). *Caenorhabditis elegans*, non-parasitic soil nematode is small measuring about 1 mm in length, optically transparent for ease of manipulation and observation. Despite having many advantages as a model organism, both the species are vaguely related to vertebrates and hence lack multifaceted immune system making them unappealing to study host-pathogen interactions and host immune responses to infection. Mammalian animal models such as mouse and rat have multifaceted immune system and human share about 98% of DNA with mice but they do not have the advantages that invertebrate model organisms have such as small size, optical transparency etc. Hence there is a need for a vertebrate animal model that possesses the qualities of an invertebrate model organism there by bridging the gap between lower animals and mammals.

1.3.1 The Zebrafish – a see-through host

As a vertebrate, the Zebrafish (*Danio rerio*) high degree of sequence and functional similarities with mammals, including humans. All proteins studied so far have a similar function in fish and mammals. Microarray and sequencing studies have shown that induction of transcriptional regulators and immune effectors are extremely preserved amongst zebrafish and human (Meijer and Spaink, 2011). List of human pathogens or closely related animal pathogens are growing rapidly. Zebrafish has become widely popular as a model for inflammatory and infectious diseases over the recent years (Allen and Neely, 2010; Sullivan and Kim, 2008). Zebrafish can be used to model many human infectious diseases either using embryos and larvae with only innate immune system or adult zebrafish with a fully developed adaptive immune system (Cui et al., 2011). It is known that cellular, biological and developmental processes are highly conserved across all vertebrates hence

better understanding of human disease processes can be obtained by studying human diseases in zebrafish. In comparison to mice, it is easier to house zebrafish in laboratory conditions due to their small size and relatively simple nature of their natural environment. Zebrafish embryos and larvae are optically transparent which offers significant advantages such as possibility to monitor the influence of a genetic manipulation, for studying host–pathogen interactions in real time by live imaging of chemotaxis, phagocytosis and pathogenesis (Cui et al. 2011) and pharmacological treatment using non-invasive imaging techniques. Furthermore, there is wide range of transgenic reporter and mutant zebrafish lines available. The zebrafish genome is fully sequenced and has been added to Genome Reference Consortium (GRC) in 2010. Number of offsprings obtained from a zebrafish pair is much larger than rodents and also offspring grow and develop very quickly. A zebrafish pair can lay 300 eggs in comparison to 5-10 obtained from rodents thereby ensuring ready supply of animals for research. It is easier to introduce genetic changes in zebrafish with latest technologies such as antisense morpholino transient gene knock down, TILLING (Targeting Induced Local Lesions IN Genomes) (Moens et al., 2008), TALENs (Transcription activator like effector nucleases) (Hwang et al., 2014) and the CRISPR/Cas system (Blackburn et al., 2013).

At larval and embryonic stages of zebrafish no adaptive immune system is developed which offers excellent opportunity to study the significance of innate immune system. At about 20 hours post fertilization primitive macrophages start to appear from the anterior lateral plate mesoderm (Herbomel et al., 1999) and neutrophils begin to emerge at 48 hours post fertilization with morphology and function similar to that of mammalian neutrophils (Levraud et al., 2009). These primitive macrophages can remove apoptotic cells, sense, counter and eliminate non-pathogenic infections and can readily phagocytose microbes from the blood circulation or tissues however neutrophils are less capable in ingesting microbes from blood compared to macrophages but can kill surface-associated bacteria very efficiently (Colucci-Guyon et al., 2011) and form neutrophil extracellular traps (NETs). First appearance of primary lymphocytes can be observed in 4 day old embryos however it takes several weeks for an adaptive immune system to be fully developed (Lam et al., 2004; Page et al., 2013) providing enough time and opportunity to study innate immune system response to an infection. Now with the availability of transgenic zebrafish lines with

fluorescent macrophages or neutrophils, progression of infection, immune cell recruitment and inflammation can be visualized in real time in the transparent zebrafish larva.

1.3.2 Microinjection and development of zebrafish infection model

Zebrafish infection model has become widely popular due to its ease of obtaining large numbers of embryos, external development, optical transparency, a wide range of genetic tools, mutant resources and transgenic reporter lines (Bernard et al., 2012). However, not all pathogens or human pathogens can cause infection in zebrafish. A pathogen should be able to overcome different defensive mechanisms of the host in order to cause an infection. Hence vulnerability of zebrafish to the pathogen of interest has to be verified before establishing an infection model. If the pathogen of interest is unable to cause infection in zebrafish, then a pathogen that is closely related to the pathogen of interest can be used. This has been magnificently attained in studying mycobacteria. *Mycobacterium marinum* which is a natural fish pathogen has been used to establish infection in zebrafish instead of *Mycobacterium tuberculosis*. This helped researcher to understand common characters of mycobacteriosis and the obtained information was later used to understand *Mycobacterium tuberculosis* infection in humans (Davies et al., 2002). Most convenient way to infect a zebrafish larva by a pathogen is by bath immersion, where pathogen is introduced directly into the water in which the zebrafish larva is kept. However due to significant variations between experiments and lack of reproducibility this technique is not always reliable.

Microinjection is a technique where a pre-adjusted number of pathogenic bacteria is artificially injected into a specific site in zebrafish embryo. Selection of injection site depends on whether the infection will become systemic or will primarily remain localized. Intravenous injection of bacteria directly into posterior blood island (Fig. 1A), the duct of Cuvier (Fig. 1B) causes systemic infection whereas injections into tail muscle (Fig. 1D), hindbrain ventricle (Fig. 1C), otic vesicle (Fig. 1E), notochord (Fig. 1F), and the yolk sac causes (initial) (Fig. 1G) local infection (Bernard et al., 2012). Bacterial injections into the yolk sac may also progress into systemic infections. Once the zebrafish vulnerability to pathogen of interest is verified, next step is to establish the infection model. Primary step is to identify appropriate injection dose i.e. minimum number of bacterial CFU required to establish an infection so that infection progresses at a slow rate without killing the host quickly. This provides enough time for the researcher to monitor infection kinetics, to study

and visualize host-pathogen interactions and to understand host immune responses to the pathogen. The role of a gene in a pathogen in promoting virulence can be studied by creating gene deletion mutants and injecting them into zebrafish. Similarly, gene deletion mutants can be created in zebrafish to identify host receptors and their contribution in combating the infection. The apparatus set up for microinjection involves a binocular stereo microscope with 10 – 40-fold magnification, a microinjection controller to control and maintain constant pressure applied to a hollow glass needle with a microscopic tip and a micromanipulator which is used to hold and lead the glass needle to the injection site.

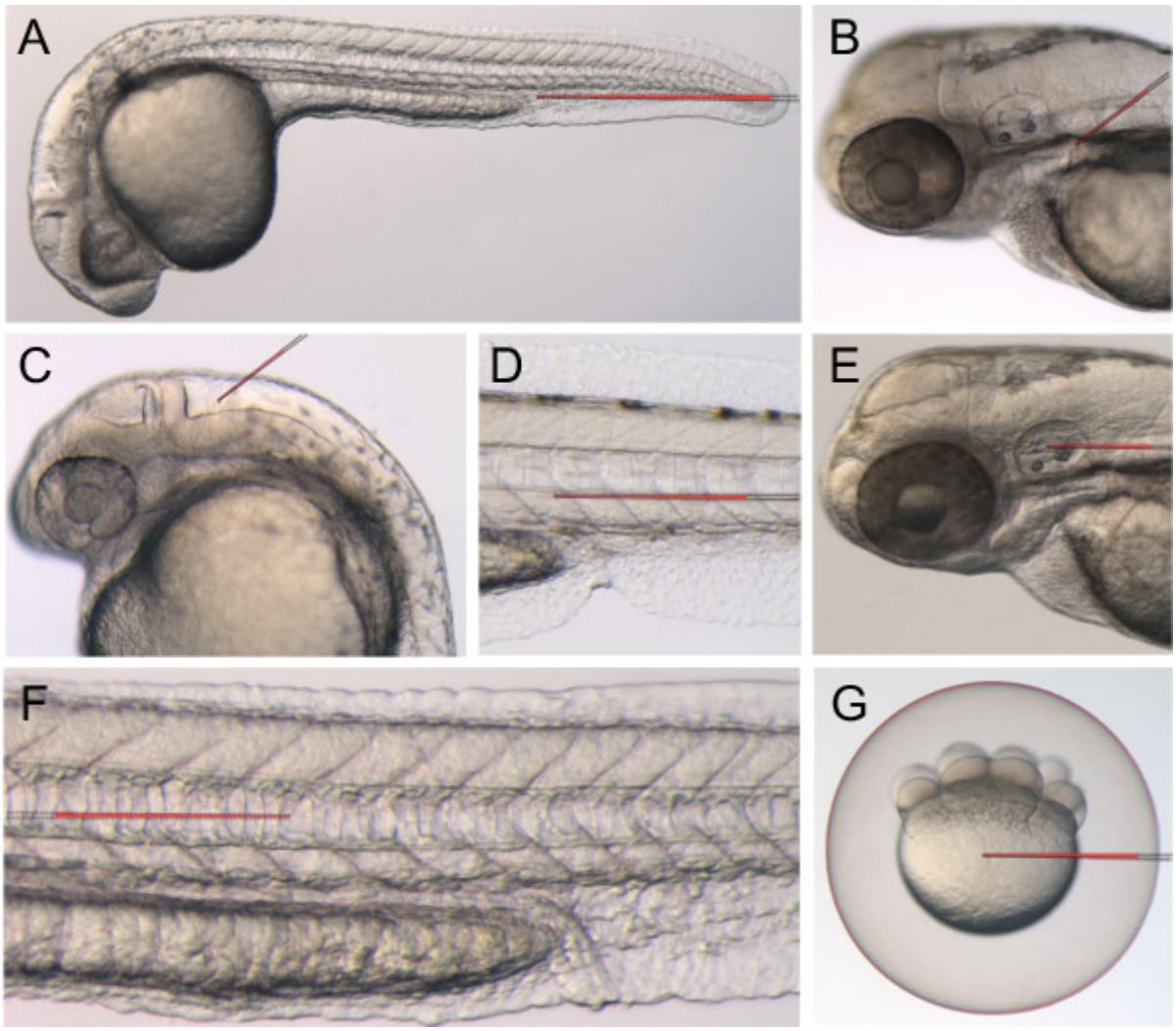

Figure 1 *Zebrafish embryo injection sites to establish either systemic or local infection. Injections directly into posterior blood island (A) or Duct of Cuvier (B) leads to a systemic infection and injections into hindbrain ventricle (C) or tail muscle (D) or otic vesicle (E) or notochord (F) or yolk sac (G) results in initial local infection. Adapted from Bernard et al., 2012.*

1.4 Pathogens of interest

Bacterial pathogens of interest in this thesis are *Cronobacter* spp. and *Listeria monocytogenes.* Both these bacteria are facultative intracellular and are capable of infecting macrophages and epithelial cells. An infection established in a transparent host like zebrafish should help us determine which of these cell types are preferentially targeted and provide better understanding of pathogenesis of these opportunistic foodborne pathogens.

1.4.1 *Cronobacter* spp.

Cronobacter are gram negative, non-spore forming bacteria belonging to the family of *Enterobacteriaceae. Cronobacter* spp. can be found almost everywhere in the environment such as soil, freshwater and even in plants. They have been frequently isolated from large variety of foods such as infant formula, processed foods, beverages, spices, fresh produce and animal products. So far, *Cronobacter* genus consists of seven species, *C. sakazakii, C. malonaticus, C. turicensis, C dublinensis, C. universalis, C. muytjensii* and *C. condimenti* (Jaradat et al., 2014). Except for *C. condimenti* all other *Cronobacter* species have been associated with clinical cases of infection in either adults or infants. *Cronobacter* infections can cause bacteremia and sepsis, meningitis and necrotizing enterocolitis in neonates, infants, immune-compromised and elderly people (Healy et al., 2010). Most often *Cronobacter* infections in adults such as bacteremia are not studied in detail due to lack of standardized procedures or because many clinical laboratories do not test for this organism. *Cronobacter* infection in infants is often linked to consumption of contaminated powdered infant formula and voluntary recalls of infant formula that were found to be contaminated with *Cronobacter* have taken place in United states and Europe. Although incidence of neonatal *Cronobacter* infections are rare, most of the cases have received considerable attention due to high mortality rate of 80 percent (Healy et al., 2010) and surviving individuals suffer from permanent sequelae and neurological problems. *Cronobacter* spp. can attach and form biofilms on the surface of plastics, glass, metals and have high tolerance to heat and drought (Lehner et al., 2005) giving it physical protection from environmental stress. Furthermore, *Cronobacter* spp. have high tolerance to acidic conditions which protect them from host defenses during infection. *Cronobacter* being a food contaminant can cause necrotizing enterocolitis in the host gut mucosa (Liu et al.,

2012) and eventually enter blood stream causing bacteremia or sepsis. Focused towards central nervous system *Cronobacter* can cross blood-brain barrier causing meningitis (Healy et al., 2010). *Cronobacter spp.* have the ability to survive and persist inside human macrophages (Eshwar et al., 2015) and about 233 putative virulence genes are found to be present in *C. turicensis* (Stephan et al., 2011). Most of the information about *Cronobacter* pathogenesis available today are from *in vitro* studies from cell culture and *in vivo* studies from neonatal rat, mouse or gerbil (Townsend et al., 2007; Mittal et al., 2009; Pagotto et al., 2009; Lee et al., 2011). Although valuable data has been acquired from these analyses, the absence of potentials for a real time examination and the need for laborious and invasive sample analysis limit the use of mammalian experimental animals. The nematode *Caenorhabditis elegans* which is known for easy rearing and transparency has also been used to study *Cronobacter* virulence factors (Sivamaruthi et al., 2011) but invertebrates are genetically not closely related to humans and lack multifaceted immune system. Hence zebrafish (*Danio rerio*) may be considered a hybrid between the mouse and invertebrate infection models. The most striking feature of this model is the possibility of performing non-invasive, high-resolution, long-term time-lapse and time-course experiments to visualize infection dynamics with fluorescent markers in the transparent embryo. Zebrafish infection model offers excellent opportunity not only to test extensive range of virulence genes but also in a high throughout manner.

1.4.2 *Listeria monocytogenes*

Listeria monocytogenes is a facultative, gram-positive, opportunistic intracellular bacterial foodborne pathogen associated with serious public health problems and significant challenges to food safety. *L. monocytogenes* is also accountable for significant financial losses to the food production industry. High-risk individuals such as immunocompromised patients and pregnant women are very susceptible to *Listeria* infection where the bacterium may cross the placental barrier and the blood-brain barrier (BBB) causing listeriosis. Listeriosis can cause spontaneous abortions and still births in pregnant women and death in 20-30% of infected immunocompromised patients (Angelidis et al., 2006; Skandamis et al., 2008). Usually, *L. monocytogenes* infects humans following the ingestion of the contaminated food such as seafood, cheeses and meat. They are also known to thrive in wide range of temperatures (Kagkli et al., 2009). *L. monocytogenes* has developed collection

of mechanisms to evade the host immune system, survive and reproduce in the host cell. Following ingestion of contaminated food *L. monocytogenes* can cross the human gut epithelium by a mechanism known as zipper mechanism where it uses Internalin A and B (InlA and B) to bind to cadherin, a protein on the intestine cell membrane (Fig. 2). These invading bacteria are then engulfed by macrophages. To survive inside the macrophages, *L. monocytogenes* uses listeriolysin O (LLO) (Fig. 2) which helps them to escape the vesicle and survive inside the macrophages (Portnoy et al., 2002). From here they can persist, reproduce and migrate to following cell subsequently disseminate to inner organs such as liver and spleen. Previous studies in mammalian models have identified few virulence factors in *L. monocytogenes* however considering array of mechanisms developed by the bacteria to evade the host immune system, there is need for a high throughout vertebrate model to analyze wide range of virulence genes and also host receptors involved during infection. In this study we have set out to examine if zebrafish could be used as a model organism to identify new virulence factors and their role in pathogenesis of *L. monocytogenes.*

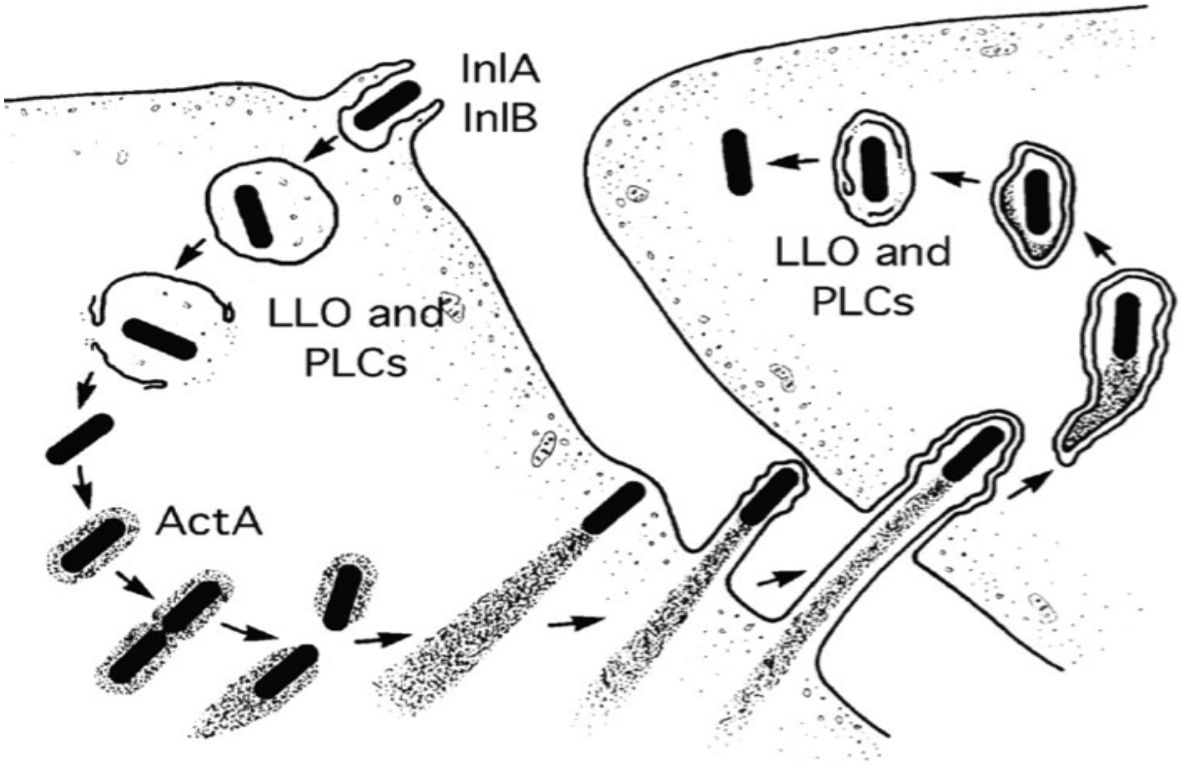

Figure 2 *Phases in the intracellular life-cycle of Listeria monocytogenes*. *Drawing portraying entry into host cell involving InlA and InlB, vacuolar lysis and escape from vacuole (LLO and PLCs), actin nucleation and actin-based motility (actA), cell-to-cell dissemination and vacuolar lysis and escape from secondary vacuole (LLO and PLCs). Adapted from Tilney and Portnoy (2002).*

1.5 Objectives of this thesis

Cronobacter spp. can cause life-threatening systemic infections, mainly in premature, low-birth weight and/or immune-compromised neonates. Underlying molecular mechanisms of disease development still remain elusive. To answer whether *Cronobacter* can survive and persist in macrophages we applied an *in vitro* phagocyte model. This model provided us an overview of the pathogen's ability to survive and replicate inside the cell but not the holistic view of the infection process in real time. Therefore, to overcome the limitations we aimed to establish and apply an *in vivo* zebrafish infection model with *Cronobacter*. Thereby we applied various approaches such as immunostaining, RNA sequencing, whole genome sequencing, microarray analysis, confocal microscopy, quantitative real time PCR, etc. to answer questions concerning pathogenesis or host immune responses to the infection.

Knowledge gained from *Cronobacter* – zebrafish infection studies motivated us to study another intracellular opportunistic pathogen, *Listeria monocytogenes* in zebrafish larval model. This study aimed for better understanding of proteins of the cold shock domain protein (Csps) family. The Csps are important regulatory proteins presumed to modulate both stress protection and virulence functions in bacteria. *L. monocytogenes* harbors three Csps (CspA, CspB and CspD). To understand various roles of Csps we created double and triple gene deletion mutants and studied them using the macrophage and the zebrafish larval model.

Chapter 2

Influence of FkpA variants on survival and replication of *Cronobacter* spp. in human macrophages

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Planning of experiments, Cell culture, Invasion and Gentamicin assays, RTPCR, statistical analysis, cell staining, confocal microscopy, image analysis, preparation of figures and graphs and writing the manuscript.

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Original article

Influence of FkpA variants on survival and replication of *Cronobacter* spp. in human macrophages

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Abstract

Members of the genus *Cronobacter* are responsible for cases of meningitis and bacteremia with high fatality rates in neonates. Macrophage uptake of invading microbes is an innate process, and it has been proposed that macrophage infectivity potentiator (Mip) like proteins enhance the ability of pathogens to survive within macrophages. *Cronobacter* harbor the *mip*-like gene *fkpA*, but its role in intracellular survival of these bacteria in human macrophages has not yet been studied. Application of gentamicin exclusion assays and human THP-1 macrophage cells revealed significant differences in the capablility of *Cronobacter* species to survive and replicate within macrophages. Analysis to the amino acid level revealed both length and sequence variations in FkpA proteins among species. In this study, we addressed the possible influence of FkpA variants in intracellular survival of *Cronobacter* spp. in human macrophages, by knocking out the *fkpA* genes in two different *Cronobacter* strains and subsequent complementation with variants of the *fkpA* genes.

Our results provide strong evidence that, in *Cronobacter* spp., FkpA must be considered a virulence factor, but its influence on macrophage survival and replication varies among strains and/or species due to the presence of amino acid variations.

Keywords: *Cronobacter* spp.; THP-1; Macrophage survival; CLSM; FkpA; Virulence factor

1. Introduction

The neonatal pathogens *Cronobacter* spp. are responsible for cases of meningitis and bacteremia, predominantly in premature and low-birth-weight infants [1]. These infections have high case fatality rates (up to 80%), with most survivors suffering long-term neurological sequelae [2]. Several reports have implicated powdered infant formula (PIF) as a possible contamination route for *Cronobacter* in neonatal infection, either from low-level intrinsic contamination of PIF or extrinsic contamination of prepared feed [3].

The genus *Cronobacter* – as proposed in 2008 – currently consists of seven species according to the "List of prokaryotic names with standing in nomenclature" (http://www.bacterio.net/allnamesac.html, viewed, 12232014) and encompasses organisms that have previously been identified as *Enterobacter sakazakii* [4,5]. The extension of the genus *Cronobacter* by three more species, as proposed by Brady et al. [6] was withdrawn as the genus membership was experimentally disproved in the recent study by Stephan et al. [7].

Information from epidemiological studies, along with in vitro mammalian tissue culture assays, has shown that *Cronobacter* isolates demonstrate a variable virulence phenotype [8–10]. Only isolates of *Cronobacter sakazakii*, *Cronobacter malonaticus* and *Cronobacter turicensis* have been linked with neonatal infections [11–13].

As an orally ingested pathogen causing systemic infections, *Cronobacter* must cross the gastrointestinal barrier and, following tropism for the central nervous system, translocate to and cross the blood–brain barrier (BBB).

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Macrophage uptake of invading microbes is an innate process as part of the first line of defense toward eliminating harmful pathogens. The ability of some bacteria to persist and replicate within these immune cells may be crucial for the survival of bacteria in the bloodstream, a primary step in development of severe illnesses such as sepsis or meningitis. Thus, the ability of *Cronobacter* to enter, persist and/or grow within macrophages is important for infection, and strains or mutants unable to survive the intracellular environment may be considered avirulent.

In studies by Townsend et al. [9,10] the persistence of *E. sakazakii* (*Cronobacter* spp.) strains was investigated, and although survival up to 96 h and replication were demonstrated for some strains, significant differences were observed among strains examined in this study.

Studies on the survival of *Salmonella* strains in murine and human macrophage cells using comparative inhibition assays indicate that persistence may be influenced by periplasmic cis–trans prolyl isomerases (PPIases) such as FkpA [14,15].

These proteins facilitate proper protein folding by increasing the rate of transition of proline residues between the cis and trans states, but may also play significant roles in survival in environmental and pathogenic niches [16]. It has been shown, that the *fkpA* genes of *Escherichia coli* and *Salmonella* are related to the *mip* (macrophage infectivity potentiator) genes of *Legionella pneumophila* and *Chlamydia trachomatis*, and involvement of the MIP proteins in the ability to survive within macrophages and epithelial cells was demonstrated for the latter intracellular pathogens [17–19].

Genbank homology searches on whole genome sequences available for several *Cronobacter* spp. strains revealed the presence of *fkpA*-like gene homologs in these organisms and the present study addresses the possible influence of FkpA variants in the intracellular survival of *Cronobacter* spp. in human macrophages.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in the study are listed in Table 1. *Citrobacter koseri* strain ATCC 25408 and *E. coli* K12 were included as controls during macrophage survival experiments. The *E. coli* DH5α::dsRED strain – used in microscopy experiments – was constructed by transformation of vector pRZT3::dsRED using standard methods.

The nalidixic-acid resistant strains *C. turicensis* LMG 23827^T_Nal^R and *Cronobacter universalis* LMG 26249^T_Nal^R, which were used in trans conjugation experiments, were constructed following the protocol of Johnson et al. [20]. *C. turicensis* LMG 23827^T::GFP was constructed during a previous study [21]. For cultivation, strains were grown in Luria–Bertani (LB) broth, overnight at 37 °C with gentle shaking. Where appropriate, culture medium or agar was supplemented with nalidixic acid at 256 μg ml^{-1} (*C. turicensis* LMG 23827^T_Nal^R, *C. universalis* LMG 26249^T_ Nal^R), chloramphenicol at 30 μg ml^{-1} (strains harboring pDS132) or tetracycline at 50 μg ml^{-1} (strains harboring pCCR9 or pRZT3::dsRED).

Table 1
Wild type strains used in the macrophage survival/replication experiments.

Species	Strain ID ATCC,[a] DSM,[b] LMG,[c] other[d]	Source and/or reference
Citrobacter koseri	ATCC 25408[d]	Baby food
Cronobacter condimenti	LMG 26250^T	Spiced meat [5]
Cronobacter dublinensis	LMG 23823^T	Milk powder [4]
Cronobacter malonaticus	LMG 23826^T	Human breast abscess [4]
Cronobacter muytjensii	ATCC 51329^T	Unknown [4]
Cronobacter sakazakii	ATCC 29544^T	Child throat [4]
Cronobacter turicensis	LMG 23827^T	Neonate [4]
Cronobacter turicensis	LMG 23827^T::GFP[d]	See above [21]
Cronobacter universalis	LMG 26249^T	Water [4,5]
Escherichia coli	K12, ATCC 27325[d]	Unknown
Escherichia coli	DH5α::dsRED	This study

[a] ATCC = American Type Culture Collection, Manassas, USA.
[b] DSM = Leibnitz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.
[c] LMG = BCCM/LMG Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.
[d] Type culture collection Institute for Food Safety and Hygiene, University Zurich, Zurich, Switzerland.

2.2. THP-1 cell culture

THP-1 cells (ATCC TIB-202) were seeded onto T75 tissue culture flasks (TPP- Techno plastic products). Cells were grown in RPMI 1640 medium (Sigma) containing 0.3 g l^{-1} L-glutamine, 2 g l^{-1} sodium bicarbonate supplemented with 10 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 4.5 g l^{-1} glucose and 10% fetal bovine heat-treated (56 °C, 30 min) serum (Sigma) and incubated at 37 °C with 5% CO_2. Twenty-four well plates were seeded with THP-1 cells at 1×10^5 cells per well and incubated in RPMI 1640 medium supplemented with 0.1 μg ml-1 of phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich) at 37 °C with 5% CO_2 for at least 24 h prior to infection. The PMA treatment has been shown to induce stable differentiation of THP-1 monocytes into macrophages without undesirable gene upregulation. This treatment caused the THP-1 cells to become adherent and activated.

2.3. Invasion/gentamicin protection assays

Prior to infection, PMA-containing medium was removed and cells were gently washed with RPMI 1640 medium to remove residual PMA and replaced with 500 μl per well of fresh medium without PMA. Bacteria for infection assays were cultured overnight in brain-heart infusion (BHI, Sigma) broth at 37 °C with gentle shaking. THP-1 macrophages were infected with an MOI of 1:100 ($\approx 10^7$ CFU per well), incubated for 45 min at 37 °C with 5% CO_2 before replacement of media with 500 μl fresh medium containing 100 μg ml^{-1} gentamicin. Plates were incubated for 45 min under the above mentioned conditions to kill extracellular bacteria.

Subsequently, infected macrophages were washed twice with 1 ml of DPBS (Dulbecco's phosphate-buffered saline). For lysis of infected macrophages, 0.5% Triton X-100 (Sigma) in DPBS was added and viable bacteria were determined via plate count on plate count (PC) agar, Sigma). Number of viable bacteria was presented as the percentage of the inoculum that is intracellular. This time point represented T0. For extended (intracellular survival/replication) assays, the 100 μg ml^{-1} gentamicin-containing media was removed, cells were washed with fresh medium and maintained in medium containing 10 μg ml^{-1} gentamicin (in experiments with wild type strains) or medium supplemented with both 10 μg ml^{-1} gentamicin and tetracycline at 50 μg ml^{-1} (in experiments with complemented mutants) during the observation period. Macrophages were incubated for 24 (T24), 48 (T48), 72 (T72) and 96 (T96) h before washing, lysis and total viable bacteria counting on PC agar (in experiments with wild type strains) or PC agar supplemented with 50 μg ml^{-1} tetracycline (in experiments with complemented mutants) as mentioned above.

For THP-1 viability, macrophages were suspended in DPBS buffer and counted by trypan blue exclusion staining using a Stereo dissecting microscope. Results are presented as percent survival of the initial intracellular population recovered at time zero for each indicated time point. For all assays, three experiments were performed on three independent occasions.

2.4. DNA extraction, manipulations, and sequencing

All kits for DNA isolation and purification were obtained from Qiagen (Hilden, Germany) and applied following the manufacturer's instructions. Chromosomal DNA was isolated using the DNeasy blood and tissue kit, plasmids were extracted with the QIAprep Spin Miniprep or plasmid midi kits. For purification purposes (PCR, restriction digest, agarose purification), the Qiagen MinElute PCR cleanup kit or MinElute gel purification kit was employed. The concentration of nucleic acids was determined using a Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Enzymes and respective buffers were obtained from Roche (Basel, Switzerland) and the reaction setup was performed following the manufacturer's instructions. All sequencing was outsourced (Microsynth, Balgach, Switzerland).

2.5. Amplification and sequence analysis of FkpA CDSs

The primer pair Cturi_univ_fkpAf (containing a *BamHI* restriction recognition site) and Cturi_univ_fkpAr (containing *HindIII* restriction recognition sites) was used to amplify *C. turicensis* LMG 23827^T as well as *C. universalis* LMG 26249^T FkpA CDSs. The amplification mix contained 0.4 μM of primers, 1× AccuPrime (Invitrogen) buffer 2 (60 mM Tris−SO_4 (pH 8.9), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, thermostable AccuPrimeTM protein, 1% glycerol), 2 U AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen) and 20 ng of template DNA. The following PCR conditions were used: 95 °C for 120 s, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s and 68 °C for 180 s, and a final elongation step at 68 °C for 300 s. Sequencing of amplicons was performed using the same primer pair.

2.6. Analysis of fkpA gene expression in macrophages

The expression levels of *fkpA* in *Cronobacter* internalized in macrophages at 48 h post infection were determined using reverse transcription quantitative-PCR (RT-qPCR). For this purpose, THP-1 macrophages were infected for 48 h with either *C. universalis* LMG 26249^T or *C. turicensis* LMG 23827^T, washed and lysed as described above. Intracellular bacteria released in these macrophage lysates were harvested in an RNAprotect bacterial reagent (Qiagen, Hilden, Germany). The recovered bacteria were re-suspended in 0.5 ml of the lysis buffer of the RNeasyPlus Mini Kit (Qiagen). The samples were transferred onto the lysing bead matrix in MagNA lyser tubes and mechanically disrupted (1 min at 6500 rpm) using the MagNA Lyser Instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland). RNA was isolated from the bacterial lysates following the RNeasy^{Plus}Mini Kit protocol (Qiagen). Genomic DNA was removed by using a genomic DNA binding column and carrying it out an on column DNAse I digestion. RNA was eluted in 50 μl of RNAse-free water, and subsequently quantified and quality controlled using the Nanodrop and BioAnalyzer instruments, respectively; 100 ng of RNA were reverse-transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). Residual DNA contamination was ruled out in each RNA sample by including a control in which the RT enzyme was omitted. Quantitative PCR was performed on 2.5 ng cDNA using the SYBR green I kit (Roche Molecular Diagnostics), and primers that are listed in Table 2 in the LC480 (Roche Molecular Diagnostics) instrument. Quantification was performed using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics). The *fkpA* mRNA levels were normalized using 16S rRNA and *fusA* as reference genes.

2.7. Construction of fkpA in frame deletion mutants for C. turicensis *LMG* 23827^T *and* C. universalis *LMG* 26249^T

The mutants were constructed following the protocol by Philippe et al. [22]. The material (bacterial strains, plasmid, primers) used for mutant construction is listed in Table 2. Primers were designed based on the whole genome sequence of *Cronobacter turicensis* LMG 23827^T (RefSeq accession numbers NC_013282 to NC_013285, GenBank accession numbers FN543093 to FN543096) and *C. universalis* LMG 26250^T (= NCTC 9529^T, WGS project CAKX01 and AJKW01 data). In brief, two fragments (one upstream (F1), one downstream (F2) of either *C. turicensis* LMG 23827^T (GenBank Accession CTU_38610) or *C. universalis* LMG 26250^T (GenBank Accession BN136_2257) CDS annotated as FkpA were amplified using primer pairs Outer_Cturi_Cuniv_fkpA_1_f (containing an *XbaI* restriction

Table 2
Material used for *fkpA* gene sequence analysis, expression studies, mutant construction and complementation experiments.

Strains/plasmids/primers	Genotype/characteristic(s)/sequences	Source or reference
FkpA gene expression analysis		
Real Time quantitative PCR primers		
Cturi_univ_16S_f	5′-GTG TTG TGA AAT GTT GGG T-3′	This study
Cturi_univ_16S_r	5′-ACT AGC GAT TCC GAC TT-3′	This study
Cturi_univ_fusA_f	5′-GGY CGT ATC GTA CAG ATG C-3′	This study
Cturi_univ_fusA_r	5′-GTC AGC TTT GGT TTT CG−3′	This study
Cturi_univ_fkpA_f	5′- CTT CCG TGA CAA RTT CGC-3′	This study
Cturi_univ_fkpA_r	5′-AAA CAC CAG RGT GGA G-3′	This study
Mutant construction		
Strains		
C. turicensis LMG 23827^T_Nal^R	Acceptor for transconjugation, Nal^R	This study
C. universalis LMG 26249^T_ Nal^R	Acceptor for transconjugation, Nal^R	This study
E. coli SM10 λpir	Host for pDS132:: Δ*fkpA* construct generation*; thi, thr, leu, tonA lacY supE recA*::RP4-2-Tc::Mu, Km, *λpir*	[24]
E. coli DH5α λpir_pDS132	Host for cloning vector pDS132; *sup* E44, Δ*lacU*169 (Φ80*lacZ*ΔM15), *recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir,* Cam^R	[25]
E. coli SM10 λpir_pDS132::CturiΔ*fkpA*	Donor for transconjugation, harbouring construct pDS132::CturiΔ*fkpA,* Cam^R	This study
E. coli SM10 λpir_pDS132::CunivΔ*fkpA*	Donor for transconjugation, harbouring construct pDS132::CunivΔ*fkpA,* Cam^R	This study
Plasmids		
pDS132	Low copy cloning vector R6K *ori, mobRP4, cat, sacB,* Cam^R	[22]
pDS132::CturiΔ*fkpA*	*C. turicensis* LMG 23827^T_Δ*fkpA* cloned into pDS132, Cam^R	This study
pDS132::CunivΔ*fkpA*	*C. universalis* LMG 26249^T_Δ*fkpA* cloned into pDS132, Cam^R	This study
Primer		
Outer_Cturi_Cuniv_fkpA_1_f	5′-TTT TTT TCT AGA AGC ATC AGA AAG TTC GTT CGC-3′	This study
Inner_Cturi_fkpA_1_r	5′-TTT TTT GTC GAC GAA CTG CTG GAT ATC AAT CCG-3′	This study
Inner_Cturi_fkpA_2_f	5′-TTT TTT GTC GAC AGC AGT ATT CTG CTG CTG TGC-3′	This study
Outer_Cturi_fkpA_2_r	5′-TTT TTT TCT AGA CTG ATG CTC ACT GAT TTC GCG-3′	This study
Control_Cturi_fkpA_f	5′-TTA TTA TAC GTG GCC CGG-3′	This study
Control_Cturi_fkpA_r	5′-AGT CTG AAA TAA CGC GCG-3′	This study
Inner_Cuniv_fkpA_1_r	5′-TTT TTT GTC GAC GAG CTG CTG GAT ATC AAT CCG-3′	This study
Inner_Cuniv_fkpA_2_f	5′-TTT TTT GTC GAC TGC GGC TTT GCT GTC AGC GGC-3′	This study
Outer_Cuniv_fkpA_2_r	5′-TTT TTT TCT AGA ATC GGC CTT GCG ATG TCG TCG-3′	This study
Control_Cuniv_fkpA_f	5′-TGC GAA GCG TTA CTT CGC-3′	This study
Control_Cuniv_fkpA_r	5′-AGT CTG AAA TAA CGC CCG-3′	This study
Complementation		
Strains		
C. turicensis LMG 23827^T	Template for amplification of CturifkpA CDS	This study
C. universalis LMG 26249^T	Template for amplification of CunivfkpA CDS	This study
C. turicensis LMG 23827^T_ Δ*fkpA*	FkpA CDS mutant, cloning host for pCCR9, pCCR9::CturifkpA, pCCR9::CunivfkpA	This study
C. turicensis LMG 23827^T_Δ*fkpA*_pCCR9	Transformant harbouring low copy cloning vector pCCR9, Tet^R	This study
C. turicensis LMG 23827^T_ Δ*fkpA*_pCCR9::CturifkpA	Transformant harbouring construct pCCR9::CturifkpA, Tet^R	This study
C. turicensis LMG 23827^T_Δ*fkpA*_pCCR9::CunivfkpA	Transformant harbouring construct pCCR9::CunivfkpA, Tet^R	This study
C. universalis LMG 26249^T_ Δ*fkpA*	FkpA CDS mutant, cloning host for pCCR9, pCCR9::CturifkpA, pCCR9::CunivfkpA	This study
C. universalis LMG 26249^T_ Δ*fkpA*_pCCR9	Transformant harbouring low copy cloning vector pCCR9, Tet^R	This study
C. universalis LMG 26249^T_Δ*fkpA*_pCCR9::CturifkpA	Transformant harbouring construct pCCR9::CturifkpA, Tet^R	This study
C. universalis LMG 26249^T_Δ*fkpA*_pCCR9::CunivfkpA	Transformant harbouring construct pCCR9::CunivfkpA, Tet^R	This study
Plasmid		
pCCR9	Low copy cloning/expression vector, Tet^R	[26]
Primer		
Cturi_univ_fkpAf	5′-CC GGA TCC ATG TTG ATT TCC GCT GCG-3′	This study
Cturi_univ_fkpAr	5′-TA AAG CTT CTC GCT AAA GTA ATA CAG-3′	This study
pCCR9-F	5′-TTT GAC AGC TTA TCA TCG-3′	[27]
pCCR9-R	5′-CCT ATG GAA GTT GAT CAG-3	[27]

recognition site) and Inner_Cturi_fkpA_1_r for amplification of F1 (containing an *SalI* restriction recognition site), Inner_Cturi_fkpA_2_f (containing an *SalI* restriction recognition site) and Outer_Cturi_fkpA_2_r (containing an *XbaI* restriction recognition site) for amplification of F2 for *C. turicensis* and Outer_Cturi_Cuniv_fkpA_1_f (containing an *XbaI* restriction recognition site) and Inner_Cuniv_fkpA_1_r (containing an *SalI* restriction recognition site) for amplification of F1 as well as Inner_Cuniv_fkpA_2_f (containing an *SalI* restriction recognition site) and Outer_Cuniv_fkpA_2_r (containing an *XbaI* restriction recognition site) for amplification of F2 for *C. universalis*. The amplification mixes contained 0.4 μM of primers, 1× AccuPrime (Invitrogen) buffer 2 (60 mM Tris−SO_4 (pH 8.9), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, thermostable AccuPrimeTM protein, 1% glycerol), 2 U AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen) and 50 ng of template DNA. The following PCR conditions were used for amplification of (*C. turicensis* and *C. universalis*) F1 fragments as well as the *C. turicensis* F2 fragment: 95 °C for 120 s followed by 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 68 °C for 180 s and a final elongation step at 68 °C for 300 s. For amplification of the *C. universalis* F2 fragment, identical conditions were used except that the annealing temperature was set to 68 °C. The fragments were double-digested using *XbaI* and *SalI* and ligated into the pDS132 vector digested with *XbaI*. The constructs pDS132::CturiΔ*fkpA* and pDS132::CunivΔ*fkpA* were transformed into *E. coli* SM10 λpir via electroporation. The resulting strains *E. coli* SM10 λpir_pDS132::CturiΔ*fkpA* and *E. coli* SM10 λpir_pDS132::CunivΔ*fkpA* served as donor strains for the subsequent transconjugation experiments using *C. turicensis* LMG 23827^T_Nal^R and *C. universalis* LMG 26250^T_Nal^R as acceptor strains, respectively. Transconjugants were selected by growth on LB agar plates supplemented with both nalidixic acid 256 μg ml^{-1} and chloramphenicol 30 μg ml^{-1}. True transconjugants were confirmed by the presence of two amplification products, one representing the chromosomal wild typ *fkpA* allele and a second product representing the truncated (Δ*fkpA*) allele originating from the (integrated) pDS132::CturiΔ*fkpA* or pDS132::CunivΔ*fkpA* vector, after PCR using primer pair Control_Cturi_fkpA_f and Control_Cturi_fkpA_r, and Control_Cuniv_fkpAf and Control_Cuniv_fkpAr, respectively, thereby employing the above mentioned AccuPrime amplification mixture and the following amplification conditions: 95 °C for 120 s, followed by 35 cycles of 95 °C for 30 s, 54 °C for 30 s, 68 °C for 180 s and a final elongation step at 68 °C for 300 s. The resulting amplification products were 1009 bp and 379 bp in size for *C. turicensis* and 1000 bp and 350 bp in size for *C. universalis*.

Outcrossing was performed by plating serial dilutions of confirmed transconjugants onto LB agar plates supplemented with 5% sucrose and without NaCl. Successful allelic exchange was verified in selected chloramphenicol-sensitive and and sucrose-resistant strains by the presence of one 379 bp (Cturi_Δ*fkpA*) or 350 bp (Cuniv_Δ*fkpA*)-sized product after amplification of chromosomal DNA using the above mentioned control primers and amplification conditions.

2.8. Complementation experiments

The primer pair Cturi_univ_fkpAf (containing a *BamHI* restriction recognition site) and Cturi_univ_fkpAr (containing *HindIII* restriction recognition sites) was used to amplify the *C. turicensis* LMG 23827^T as well as *C. universalis* LMG 26249^T *fkpA* CDSs following the procedure described in the section "Amplification and sequence analysis of FkpA CDSs".

The amplicons were double-digested with restriction enzymes *Hind*III and *Bam*HI and cloned into the low copy vector pCCR9, which had been digested with the respective enzymes to create complementation vectors pCCR9::CturifkpA and pCCR9::CunivfkpA. Both of the constructs were transformed into the *C. turicensis* LMG 23827^T_Δ*fkpA* mutant strain, thereby creating strains *C. turicensis* LMG 23827^T_Δ*fkpA*_pCCR9::CturifkpA and *C. turicensis* LMG 23827^T_Δ*fkpA*_pCCR9::CunivfkpA, respectively, as well as into *C. universalis* LMG 26249^T_Δ*fkpA*, thus resulting in strains *C. universalis* LMG 26249^T_Δ*fkpA*_pCCR9::CturifkpA and *C. universalis* LMG 26249^T_Δ*fkpA*_pCCR9::CunivfkpA. Additionally, strains *C. turicensis* LMG 23827^T_Δ*fkpA* and *C. universalis* LMG 26249^T_Δ*fkpA*_ were transformed with the pCCR9 vector only (no inserts). The resulting strains *C. turicensis* LMG 23827^T_Δ*fkpA*_pCCR9 and *C. universalis* LMG 26249^T_Δ*fkpA*_pCCR9 served as controls. All transformations were carried out via electroporation and transformants were selected on LB agar supplemented with tetracycline at 50 μg ml^{-1}. The presence of the respective *fkpA* genes on the pCCR9 vector was confirmed by amplification and sequencing of the inserts using pCCR9-F and pCCR9-R (data not shown).

2.9. Confocal laser scanning microscopy

Strains *C. turicensis* LMG 23827^T::GFP and *E. coli* DH5α::dsRED were used in macrophage infection assays with subsequent microscopic analysis. Sterile glass coverslips (13 mm in diameter, Menzel-Gläser) were placed in each well of the 24-well plate and THP-1 cells were seeded on the coverslips. Cell culture, PMA activation and infection assays were carried out was mentioned above. For each indicated time point, THP-1 macrophages adhering to coverslips were gently washed twice with DPBS or RPMI medium and fixed with 4% paraformaldehyde (PFA, Sigma) at 4 °C for 15 min. After fixation, macrophages were gently washed with DPBS to remove residual PFA and stained with 3.5 μl of 3 μg ml^{-1} Hoechst Dye (Life Technologies, Zug, Switzerland) and 5 μl of 5 mg ml^{-1} Concanavalin A Alexa Fluor® 488 Conjugate (Life Technologies) or Concanavalin A Alexa Fluor® 594 Conjugate (Life Technologies), depending on whether bacteria used for infection assay were GFP- or dsRed-expressing, and incubated at room temperature for 1 h. After incubation, macrophages were washed 3−5 times with DPBS, mounted on glass slides using Fluoromount (Sigma) mounting medium,

air-dried in the dark and imaged under a Leica TCS SP5 confocal microscope (63× or 40× oil-immersion objective, excitation at 405 nm for Hoechst, 488 nm for GFP, 592 nm for dsRED, and 488 or 594 nm for Concanavalin A, respectively).

2.10. Statistical analysis

The number of intracellular bacteria after 45 min co-incubation with macrophages, followed by 2 h 100 μg gentamicin treatment is presented as time point zero (T0). Long-term survival of bacteria within macrophages was assessed by performing a gentamicin protection assay at several time points up to 96 h (T96). Results are presented as percentage of initial inoculum that was intracellular at time point zero. Data are means ± standard error of three independent assays performed in triplicate.

fkpA gene expression data from *C. universalis* LMG 26249^T and *C. turicensis* LMG 23827^T strains were log-converted and compared using *t*-tests, where *P*-values <0.05 were considered to be statistically significant.

3. Results and discussion

Human THP-1 macrophages were utilized to examine the capacity of the strains to persist and replicate within human macrophages following phagocytosis. All *Cronobacter* species tested were able to replicate within macrophages, although this ability differed among strains (Fig. 1). *C. turicensis* LMG 23827^T (along with the GFP variant of this strain) exhibited significantly higher replication and survival capability than all other strains/species (Fig. 1). Generally, replication reached its peak after 48 h (T48) where the highest bacterial load was detected in macrophage cells. All strains except *C. universalis* LMG 26249^T were capable of persisting within macrophages up to 96 h. Two controls were included in these assays; while *E. coli* K12 was killed by macrophages, *C. koseri* strain ATCC 25408 continued to replicate until the end of the observation period.

Using the GFP variant of strain *C. turicensis* LMG 23827^T and *E. coli* DH5α::dsRED, we were able to visualize the behavior of the strains during the time course of macrophage survival/replication assays (Fig. 2). Continuous replication was observed for *C. turicensis* LMG 23827^T::GFP, reaching its peak at 48 h (Fig. 2A–C); however, even though fewer bacteria were internalized in the macrophages at T72 than at T48, some of them still seemed to replicate (Fig. 2D). A totally different result was observed during the *E. coli* DH5α::dsRED macrophage infection assays: while a bacterial number comparable to that of *C. turicensis* LMG 23827^T::GFP assays was observed at T0 (Fig. 2A, E), almost all *E. coli* were cleared at T24 (Fig. 2F). These results fit with quantitative data presented in Fig. 1.

In some bacterial species, periplasmic cis–trans prolyl isomerases have been shown to play a role in virulence and have been termed macrophage infectivity potentiators (Mip) [14,19,23]. The *fkpA* gene of *E. coli* is related to the *mip* genes

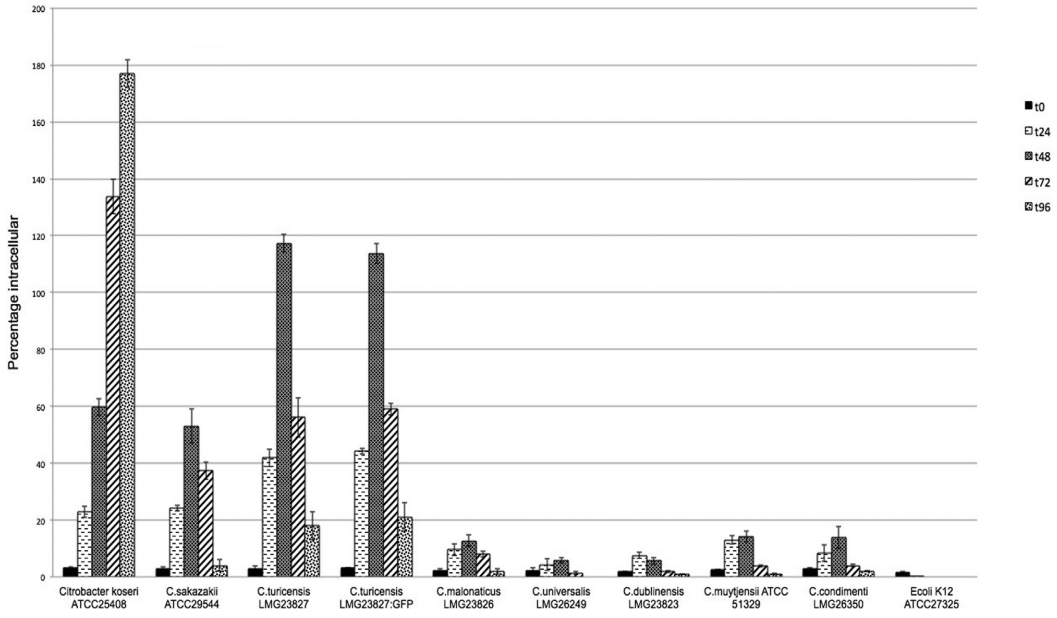

Fig. 1. Survival and replication of *Cronobacter* spp. type strains and control strains within THP-1 macrophages. The gentamicin protection assay was performed on phagocytotic THP-1 macrophages using mid-exponential-phase bacteria at various time points up to 96 h (T96). Results are presented as the percentage of the initial inoculum that was intracellular. Data are means ± SEM from three assays performed in triplicate; *E. coli* K-12 (killed) and *Citrobacter koseri* strain ATCC 25408 (replicates up to 96 h) were used as controls. Strain *C. turicensis* LMG 23827^T::GFP was included in macrophage infection experiments in order to demonstrate that this variant behaves in a manner analogous to that of the wild type strain.

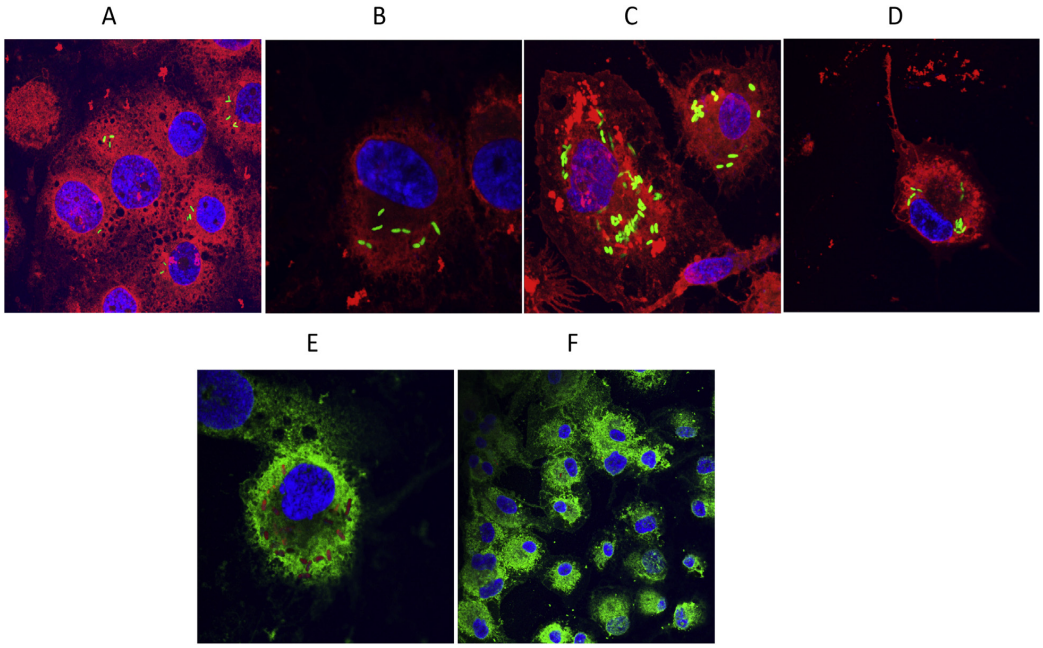

Fig. 2. Microscopic analysis of *C. turicensis* LMG 23827^T::GFP (green) and *E. coli* DH5α::dsRED (red) during THP-1 infection assays. Macrophages were stained with Hoechst dye (blue, nucleus stain) and Concanavalin A (Con) (lectin stain). Con A Alexa Fluor® 488 conjugate (green) was used in *E. coli* DH5α::dsRED experiments and Con A Alexa Fluor® 594 conjugate (red) was used in *C. turicensis* LMG 23827^T::GFP assays. Representative images of *C. turicensis* LMG 23827^T::GFP infected macrophages at T0 (A, scale bar 10 μm, 40× magnification), T24, (B, scale bar 5 μm, 63× magnification), T48, (C, scale bar 5 μm, 63× magnification) and T72 (D, scale bar 10 μm, 63× magnification). Results for *E. coli* DH5α::dsRED macrophage infection assays are depicted for T0 (E, scale bar 5 μm, 63× magnification) and T24 (F, scale bar 20 μm, 40× magnification).

of *L. pneumophila* and *C. trachomatis*, and similar genes are present in several representatives of the *Enterobacteriaceae*, some of which are not intracellular pathogens [17]. In an effort to elucidate possible reasons for the striking differences in survival and replication capacities among *Cronobacter* strains, as observed in this study and encouraged by the fact that *fkpA* homologes are present in several *Cronobacter* strains for which whole genome sequences are available, we decided to focus on this protein and its possible involvement during macrophage survival experiments. Strains *C. turicensis* LMG 23827^T and *C. universalis* LMG 26249^T were selected, as these two strains represented the two extremes, i.e. *C. turicensis* LMG 23827^T represented excellent survival and replication, while *C. universalis* LMG 26249^T represented least survival and replication, according to results of macrophage survival assays performed in the first part of the study (Fig. 1).

In a first step, *fkpA* CDSs of the two relevant strains were amplified, sequenced and analyzed at the amino acid level. In Fig. 3, amino acid alignment of the FkpA sequences as retrieved from Genbank (small letters) and translated sequences of the *fkpA* amplicons of the respective strains (capital letters) is shown. The two coding sequences differ in the length of the protein, but are otherwise identical. While *C. turicensis* LMG 23827^T FkpA consists of 275 amino acids, that of *C. universalis* LMG 26249^T is composed of 276 amino acids. The respective aa position is marked with a red arrow in Fig. 3.

In a second step, we checked for the *fkpA* gene expression of *C. universalis* LMG 26249^T and *C. turicensis* 23827^T strains during growth in macrophages using RT-qPCR. As shown in Fig. 4, despite clear differences observed in macrophage survival capacity, the two strains had similar *fkpA* mRNA levels at 48 hpi of the THP-1 macrophages. This suggested that differences observed in macrophage survival between these two strains was not associated with differences in *fkpA* gene expression.

Thus, we hypothesized that variations in FkpA protein sequences might contribute to the observed differences in survival/replication capacities of the two *Cronobacter* strains.

In order to test this hypothesis, *fkpA* genes of *C. turicensis* LMG 23827^T as well as *C. universalis* LMG 26249^T were knocked out by creating in-frame deletion mutants. The parent strains or mutant strains were used to infect phagocytotic THP-1 macrophage cells. The numbers of parent bacteria or mutant bacteria recovered at T0 were similar. However, significantly fewer mutant bacteria were recovered from cells at T24, T48, T72 and T96 hpi in the mutants (Fig. 5). In addition, while the intracellular numbers of the parent strain increased >14-fold and >35-fold over 24 h and 48 h, respectively, reduced replication rates of ≤10-fold at T24 and 21-fold at T48 were observed in the mutant strains (Fig. 5). Reintroduction of the *C. turicensis* LMG 23827^T wild-type *fkpA* gene in trans restored the ability of the mutant to survive and grow within the cell lines to a large extent (Fig. 5). In

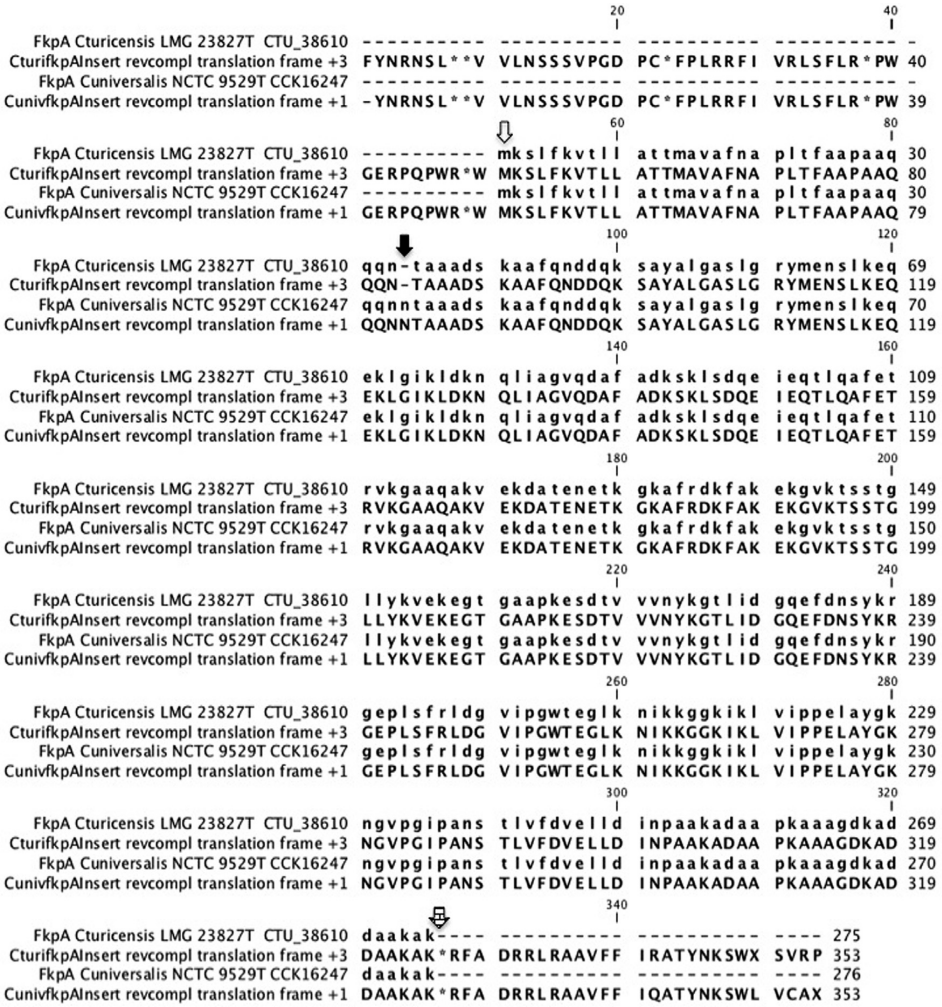

Fig. 3. Amino acid sequence alignment of *C. turicensis* LMG 23827^T (CTU_38610) and *C. universalis* NCTC 9529^T (CCK16247) wild type (aa in small letters) and cloned (this study, aa in capital letters) FkpA. Start codon (M, m) is labelled with a white arrow; the amino acid position showing relevant differences between FkpA sequences of the two different strains is labelled by a black arrow; stop codon is marked by a dashed arrow.

contrast, complementation of the *C. turicensis* LMG 23827^T mutant strain with the *fkp*A gene originating from *C. universalis* LMG 26249^T did not enhance survival or replication capacity during macrophage experiments to the same extent; rather, values remained at levels comparable to that of the mutant or the mutant transformed with the (empty) vector (Fig. 5).

This difference in survival/replication capacity could also, although to a lesser extent, be observed when the two variants of the *Cronobacter fkpA* genes were introduced into the *C. universalis* LMG 26249^T_Δ*fkpA* background. While the invasion rate (number of bacteria recovered at T0) was comparable in transformed (and untransformed) *C. universalis* strains, the *C. universalis* LMG 26249^T_Δ*fkpA* strain containing *C. turicensis* LMG 23827^T *fkpA* showed enhanced capacity to replicate (T24) and survive up to 48 h in macrophages when compared to the *C. universalis* LMG 26249^T wild type and/or the *C. universalis* LMG 26249^T_Δ*fkpA* complemented with the *C. universalis fkpA* gene (Fig. 5). It is noteworthy that a low copy vector was used for all complementation experiments and the inserted *fkpA* gene (s) is/are present in single (low) copy number(s), which reflects a situation close to the natural condition in *Cronobacter* spp.

The role of FkpA with respect to virulence has been studied in *Salmonella* serovar Typhimurium [14,15], but results were contradictory. While, in the study by Horne et al. [14], inactivation of the *fkpA* gene of *Salmonella* serotype Typhimurium resulted in reduced survival capacity in macrophages and epithelial cells, no such effect was observed in the study by Humphreys et al. [15]. The authors of the latter study hypothesized that this may be due to the different genetic

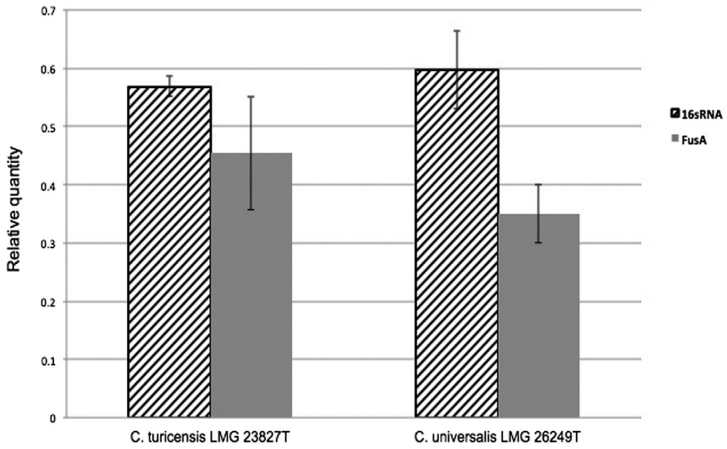

Fig. 4. RT-qPCR analysis of *fkpA* gene expression in *C. turicensis* LMG 23827^T and *C. universalis* LMG 26249^T strains grown in THP-1 macrophages at 48 h post infection. *fkpA* transcripts were normalized to 16S rRNA and *fusA* reference genes. *fkpA* mRNA levels presented are expressed relative to that of the BHI-medium-grown stationary phase *C. universalis* LMG 26249^T strain. Results are means (bars) and standard deviations (error bars) of three independent biological experiments.

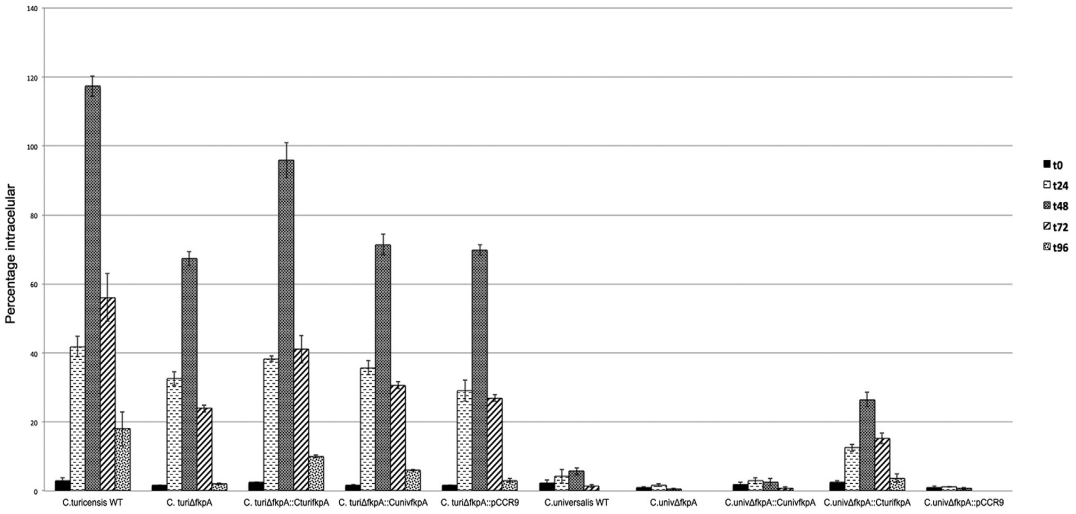

Fig. 5. Results of macrophage survival assays on *C. turicensis* LMG 23827^T and *C. universalis* LMG 26249^T wild type strains, respective Δ*fkpA* mutants and mutants complemented with either of the two *fkpA* genes. *C. turicensis* LMG 23827^T_Δ*fkpA*_pCCR9 and *C. universalis* LMG 26249^T Δ*fkpA*_pCCR9 served as controls. The following strain codes in the figure correspond to strain names as they appear in Table 1 and throughout the text: *C. turicensis* WT = *C. turicensis* LMG 23827^T; C. turiΔfkpA = *C. turicensis* LMG 23827^T_Δ*fkpA*; C. turiΔfkpA::CturifkpA = *C. turicensis* LMG 23827^T_Δ*fkpA*_pCCR9::CturifkpA; C. turiΔfkpA::CunivfkpA = *C. turicensis* LMG 23827^T_Δ*fkpA*_pCCR9::CunivfkpA; C. turiΔfkpA::pCCR9 = *C. turicensis* LMG 23827^T_Δ*fkpA pCCR9*; *C. universalis* WT = *C. universalis LMG 26249^T*; C. univΔfkpA::CturifkpA = *C. universalis LMG 26249^T ΔfkpA*_pCCR9::CturifkpA; C. univΔfkpA::CunivfkpA = *C. universalis LMG 26249^T ΔfkpA*_pCCR9::CunivfkpA; C. univΔfkpA = *C. universalis* LMG 26249^T Δ*fkpA*; C. univΔfkpA::pCCR9 = *C. universalis* LMG 26249^T Δ*fkpA*_pCCR9.

backgrounds of the bacterial strains used in the two studies. Unfortunately, FkpA of the strains used in the two studies was not analyzed at the amino acid level.

Results of this study can be summarized in the following manner: (1) FkpA contributes to the ability of *Cronobacter* species to survive and replicate within macrophages and thus must be designated a virulence factor; (2) the scale of this contribution seems to be related to FkpA protein sequence variations observed among species. Further studies will be required in order to elucidate a possible correlation between FkpA protein sequence variations and macrophage survival capacity in *Cronobacter* spp.

Conflict of interest

The authors have no financial support or relationships.

Acknowledgments

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Chapter 3

Evaluation of zebrafish as a model to study the pathogenesis of the opportunistic pathogen *Cronobacter turicensis*

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Contribution:

Planning of experiments, microinjection experiments, bacterial enumeration by plate counting, statistical analysis, Fluorescence and confocal microscopy, image analysis, live imaging, preparation of figures 1E, 1F, 2, 4, 5B, 5C, 6 and writing the manuscript.

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ORIGINAL ARTICLE

Evaluation of zebrafish as a model to study the pathogenesis of the opportunistic pathogen *Cronobacter turicensis*

Alexander Fehr[1,*], Athmanya K Eshwar[2,*], Stephan CF Neuhauss[3], Maja Ruetten[1], Angelika Lehner[2] and Lloyd Vaughan[1]

Bacteria belonging to the genus *Cronobacter* spp. have been recognized as causative agents of life-threatening systemic infections, primarily in premature, low-birth weight and/or immune-compromised neonates. Knowledge remains scarce regarding the underlying molecular mechanisms of disease development. In this study, we evaluated the use of a zebrafish model to study the pathogenesis of *Cronobacter turicensis* LMG 23827^T, a clinical isolate responsible for two fatal sepsis cases in neonates. Here, the microinjection of approximately 50 colony forming units (CFUs) into the yolk sac resulted in the rapid multiplication of bacteria and dissemination into the blood stream at 24 h post infection (hpi), followed by the development of a severe bacteremia and larval death within 3 days. In contrast, the innate immune response of the embryos was sufficiently developed to control infection after the intravenous injection of up to 10^4 CFUs of bacteria. Infection studies using an isogenic mutant devoid of surviving and replicating in human macrophages (Δ*fkpA*) showed that this strain was highly attenuated in its ability to kill the larvae. In addition, the suitability of the zebrafish model system to study the effectiveness of antibiotics to treat *Cronobacter* infections in zebrafish embryos was examined. Our data indicate that the zebrafish model represents an excellent vertebrate model to study virulence-related aspects of this opportunistic pathogen *in vivo*.
Emerging Microbes and Infections (2015) **4,** e29; doi:10.1038/emi.2015.29; published online 27 May 2015

Keywords: *Cronobacter turicensis*; pathogenesis; zebrafish model

INTRODUCTION

Cronobacter spp. are regarded as opportunistic facultative intracellular pathogens associated with the ingestion of contaminated reconstituted infant formula and cause serious illness predominantly in low-birth-weight preterm and neonatal infants.[1,2] The clinical presentation of *Cronobacter* infections include necrotizing enterocolitis (NEC), bacteremia and meningitis, with case fatality rates ranging between 40% and 80%.[3,4]

The genus *Cronobacter* spp. - as proposed in 2008 - currently consists of seven species according to the "List of prokaryotic names with standing in nomenclature" and encompasses organisms that have previously been identified as *Enterobacter sakazakii*.[5,6,7] Recently, the extension of the genus *Cronobacter* by three more *Enterobacter* species was proposed by Brady *et al.*[8]; however, re-examination of the biological basis for this suggestion as performed in the study by Stephan *et al.*[9] does not support further revision of the taxon at this time.

Epidemiological studies and *in vitro* mammalian tissue culture assays have shown that *Cronobacter* isolates demonstrate a variable virulence phenotype. To date, only isolates of *C. sakazakii*, *C. malonaticus* and *C. turicensis* have been linked to infantile infections.[10]

Despite the progressive increase in research on *Cronobacter* pathogenesis in the last decade, knowledge of the exact mechanisms of infection remains fragmentary.[11]

As an orally transmitted pathogen, *Cronobacter* is thought to gain entrance into the human body through the gastrointestinal tract, where it may cause NEC or, by unknown mechanism(s), may enter the systemic circulation without the manifestation of NEC.[12] Once the bacteria have entered the blood stream, they exhibit a tropism towards the central nervous system, showing an increased propensity to cause meningitis among low-birth-weight neonates and infants, while causing bacteremia or sepsis among slightly higher birth-weight infants.[13] After crossing the blood brain barrier, the pathogen enters the brain, where it causes ventriculitis, which can lead to the development of hydrocephalus, or forms other sequelae, such as cysts or brain abscesses.[3,14]

Most of the data available today have been acquired from *in vitro* studies. *In vivo* studies to confirm and extend these observations from cell culture have largely been concentrated on the neonatal rat, mouse or gerbil as model organism.[15-18] Although valuable information has been obtained from these studies, the lack of possibilities for a real-

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time analysis and the need for laborious and invasive sample analysis limit the use of mammalian experimental animals.

The nematode *Caenorhabditis elegans* has been alternatively used to study *Cronobacter* virulence factors, exploiting the amenities of the nematode system, such as easy cultivation and transparency.[19] However, invertebrates are genetically not closely related to humans, and their immune system shows many differences from the human immune system. Hence, other models are needed to address specific questions related to the innate immune response to a specific pathogen in detail.

The zebrafish (*Danio rerio*) may be considered a hybrid between the mouse and invertebrate infection models. The most impressive feature of this model is the possibility of performing non-invasive, high-resolution, long-term time-lapse and time-course experiments to visualize infection dynamics with fluorescent markers in the transparent embryo. The small size, ease of breeding, high fertility and genetic tractability of the zebrafish are further favorable features that make the zebrafish embryo an attractive model organism. Furthermore, the zebrafish immune system displays many similarities to that of mammals, with counterparts for most of the human immune cell types.[20] The zebrafish innate immune system starts to develop as early as 24 h post fertilization (hpf) with primitive macrophages followed by neutrophils at 32-48 hpf. The development of the adaptive immune system lags,[21] which provides an opportunity to study independently the innate immunity of the larvae during the first days post fertilization (dpf). This situation sets zebrafish apart from both *in vitro* and mammalian *in vivo* infection models. Zebrafish larvae have previously been used to study infections of other bacterial pathogens,[22] including *Listeria monocytogenes*, *Salmonella* Typhimurium and *Shigella flexneri*.[23-25]

In this study, we exploited the advantages of the zebrafish to investigate infections by *Cronobacter turicensis* LMG 23827^T *in vivo*. We show here that *Cronobacter* causes lethal infection in zebrafish larvae, with similarities to human cases. After having successfully established the experimental parameters, the model was evaluated using a strain devoid of a gene that has recently been described as a virulence factor in *C. turicensis*. In addition, the model was used to study the effectiveness of different antibiotics to treat *Cronobacter* infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *C. turicensis* LMG 23827^T, a clinical isolate responsible for the death of two neonates in Zurich in 2006 has been object of previous research.[26,29-31], Construction of the *C. turicensis* LMG 23827^T Δ*fkpA* mutant, the complemented mutant *C. turicensis* LMG 23827^T Δ*fkpA*::*fkpA*Tet^R, as well as the mutant carrying the complementation vector pCCR9Tet^R only (*C. turicensis* LMG 23827^T Δ*fkpA*::pCCR9Tet^R) has been described in detail in the study by Eshwar *et al.*[28]

The green fluorescent protein (GFP)-expressing strain *C. turicensis* LMG 23827^T::GFPKan^R was constructed by Schmid *et al.*[27] For selection purposes, during zebrafish embryo infection experiments, *C. turicensis* LMG 23827^T::dsREDTet^R, *C. turicensis* LMG 23827^T Δ*fkpA*::dsREDTet^R as well as *E. coli* DH5α::dsREDTet^R were constructed in this study by transformation of vector pRZT3::dsREDTet^R using standard methods. Plasmid pRZT3::dsREDTet^R carrying the red fluorescent protein was a kind gift by A. M. van der Sar (VU University Medical Center, Department of Medical Microbiology and Infection Control, Netherlands).

For cultivation, the strains were grown in 10 mL of Luria–Bertani (LB, Difco, Beckton, Dickinson and Company, Allschwil, Switzerland) broth overnight at 37 ℃ with gentle shaking. *C. turicensis* LMG 23827^T variants/mutants were cultivated in LB broth supplemented where appropriate with either tetracycline at 50 mg/L or kanamycin at 50 mg/L.

For microinjection experiments, the bacteria were harvested by centrifugation at 5000*g* for 10 min and washed once in 10 mL of Dubelccòs phosphate buffered saline (DPBS, Life Technologies, Zug, Switzerland. After a second centrifugation step, the cells were resuspended in DPBS, and appropriate dilutions were prepared in DPBS.

Zebrafish lines and husbandry

Zebrafish (*Danio rerio*) strains used in this study were predominantly *albino* lines as well as transgenic fish of the *Tg(lyz:DsRED2)nz50* line that produce red fluorescent protein in neutrophils, received as a kind gift from Professor Philip Crosier, University of Auckland (New Zealand).[32] Adult fish were kept at a 14/10-h light/dark cycle at a pH of 7.5 and 27 ℃. Eggs were obtained from natural spawning between adult fish which were set up pairwise in separate breeding tanks. Embryos were raised in petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) supplemented with 0.3 mg/L of methylene blue at 28 ℃. From 24 hpf, 0.003 % 1-phenyl-2-thiourea (PTU, Sigma-Aldrich, Buchs, Switzerland) was added to prevent melanin synthesis. As *albino* lines lack melanized chromophores, no PTU treatment was performed on these lines. Embryo staging was performed according to Kimmel *et al.*[33]

Research was conducted with approval (NO 216/2012) from the Veterinary Office, Public Health Department, Canton of Zurich (Switzerland).

Microinjection experiments

Injections were conducted using borosilicate glass microcapillary injection needles (1 mm outside diameter×0.78 mm inside diameter,

Table 1 Bacterial strains and mutants used in this study

Strain/mutant	Description	Source and/or reference
Cronobacter turicensis LMG 23827^T	Wild type	Neonate, Essers *et al.*[26]
Cronobacter turicensis LMG 23827^T::GFP	GFP-expressing, Kan^R	Schmid *et al.*[27]
Cronobacter turicensis LMG 23827^T::dsRED	Harboring pRZT3::dsRED, Tet^R	This study
Cronobacter turicensis LMG 23827^T Δ*fkpA*	In-frame mutant in *mip*-like gene	Mutant collection Institute for Food Safety and Hygiene, Eshwar et al.[28]
Cronobacter turicensis LMG 23827^T Δ*fkpA* ::dsRED	In-frame mutant in *mip*-like gene, harboring pRZT3::dsRED, Tet^R	This study
Cronobacter turicensis LMG 23827^T Δ*fkpA::fkpA*	Mutant complemented with wt *fkpA* gene, Tet^R	Eshwar *et al.*[28]
Cronobacter turicensis LMG 23827^T Δ*fkpA*::pCCR9	Mutant harboring empty pCCR9 vector, Tet^R	Eshwar *et al.*[28]
Escherichia coli DH5α::dsRED	Harboring pRZT3::dsRED, Tet^R	This study

Science Products GmbH, Hofheim, Germany) and a PV830 Pneumatic PicoPump (World Precision Instruments, Sarasota, Florida, USA). Prior to injection, embryos of 2 dpf were manually dechorionated and anesthetized with 200 mg/L buffered tricaine (Sigma-Aldrich, Buchs, Switzerland). Afterwards, the embryos were aligned on an agar plate and injected with 50 to 10^4 colony forming units (CFUs) in a 1-2-nL volume of a bacterial suspension in DPBS either directly into the blood circulation, the hindbrain ventricle or into the yolk sac. The volume of the injected suspension was previously adjusted by injection of a droplet into mineral oil and measurement of its approximate diameter over a scale bar. To determine the actual number of CFU injected, we initially injected inoculum directly onto the agar plates; however, a more precise determination of the injected CFU can be obtained by plating five embryos separately immediately after microinjection (0 hpi).

After injection, the infected embryos were allowed to recover in a petri dish with fresh E3 medium for 15 min. To follow the infection kinetics, the embryos were transferred to 6-well plates in groups of approximately 15 embryos in 4 mL of E3 medium per well, incubated at 28 ℃ and observed for signs of disease and survival under a stereomicroscope twice a day.

Five embryos or larvae were collected at each time point, generally 0, 15, 24, and 48 hpi, and independently treated for bacterial enumeration. The sampled larvae were euthanized with an overdose of 4 g/L buffered tricaine and transferred to different buffers and fixatives for subsequent analyses.

Bacterial enumeration by plate counting

The larvae were transferred to 1.5-mL microfuge tubes and disintegrated by repeated pipetting and vortexing for 3 min in 1 mL of PBS supplemented with 1 % Triton X- 100 (Sigma-Aldrich, Buchs, Switzerland). Subsequently, 100 µL of this mixture was plated onto LB selective plates (i.e., tetracycline 50 mg/L for strains harboring pCCR9 or pRZT3::dsRED or kanamycin 50 mg/L for selection for *C. turicensis* LMG 23827^T::GFP). The plates were incubated up to 48 h at 37 ℃.

For survival assays, the embryos were similarly microinjected and maintained individually in 24-well plates in E3 medium at 28 ℃. At regular time points after infection, the number of dead larvae was determined visually based on the absence of a heartbeat.

Drug testing

Uninfected embryos were tested for antibiotic toxicity prior to the start of this experiment by incubating embryos in E3 medium supplemented with one of three drugs. For drug screening, the infected embryos were transferred into 24-well plates after yolk injections with one embryo in each well in 1 mL of E3 medium containing either 8 mg/L ampicillin, 8 mg/L tetracycline, 4 mg/L nalidixic acid or were left untreated (no drug added). As an additional control, a set of uninfected embryos was incubated in E3 medium to determine embryo quality. A negative control group was injected with DPBS. Drugs were added to the water at the required concentrations. Samples were collected and analyzed as described above.

Statistical analysis

Statistics and graph design were performed using GraphPad Prism 6 (GraphPad Software, San Diego, United States). Experiments were performed at least three times, unless stated otherwise. The CFU of groups of individual larva at various time points and under various conditions were tested for significant differences by one-way ANOVA with Bonferroni's post-test.

Light microscopy, fluorescence imaging and image analysis

For histological examination, whole zebrafish larvae were fixed in 4% paraformaldehyde at 4 ℃ and embedded in cubes of cooked egg white to position them correctly for histological sections. These cubes containing the larvae were dehydrated in an alcohol series of ascending concentrations ending in xylene and afterwards embedded in paraffin. Paraffin blocks were cut in 2-3-µm thin sections, mounted on glass slides and stained using a routine protocol with hematoxylin and eosin. Histological imaging was performed with a Leica DM LS S-203675 (Leica microsystems, Heerbrugg, Switzerland) upright light microscope.

Overview images of whole larvae were obtained with an Olympus BX61 upright light microscope with both bright field and fluorescence modules. The fluorescence filter cube used was optimized for DAPI/FITC/TRIC. For higher resolution images, 3D-image stacks of whole mount samples were prepared using a confocal laser-scanning microscope (CLSM, Leica TCS SP5, Leica Microsystems, Heerbrugg, Switzerland). GFP, dsRED and DAPI were sequentially excited with the 405 nm, 488 nm and 561 nm laser lines, respectively, with emission signals collected within the respective range of wave lengths. 3D image stacks were collected sequentially (to prevent blue-green–red channel cross-talk) according to Nyquist criteria and deconvolved using HuygensPro via the Huygens Remote Manager v2.1.2 (SVI, Netherlands). Images were further analyzed with Imaris 7.6.1 (Bitplane, Zurich, Switzerland) and aligned with Adobe Photoshop Elements 12.

Transmission electron microscopy

For transmission electron microscopy (TEM), the larvae were fixed in a mixed solution of 1 % paraformaldehyde (Sigma-Aldrich, Buchs, Switzerland) and 2.5 % glutaraldehyde (Sigma-Aldrich, Buchs, Switzerland) in 0.1 M sodium phosphate buffer, pH 7.5 at 4 ℃ overnight. Afterwards, the samples were prepared for embedding into epoxy resin and for transmission electron microscopy according to standard procedures. Pospischil *et al.*, 1990). Epoxy resin blocks were screened for larvae using semithin sections (1 µm), which were stained with toluidine blue (Sigma-Aldrich, Buchs, Switzerland) to visualize tissue. Ultrathin sections (80 nm) were mounted on copper grids (Merck Eurolab AG, Dietlikon, Switzerland), contrasted with uranyl acetate dihydrate (Sigma-Aldrich, Buchs, Switzerland) and lead citrate (Merck Eurolab AG, Dietlikon, Switzerland) and investigated using a Philips CM10 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands). Images were processed with Imaris (Bitplane AG, Zurich, Switzerland) and assembled for publication using Adobe Photoshop.

Confocal live imaging

To perform high-resolution confocal live imaging, the injected larvae were positioned in 35-mm glass-bottom dishes (Iwaki, Eurodyne Limited, Lindale, UK). The entire larva was covered and immobilized with 1 % low-melting-point agarose solution. A total of 2 mL of fish water containing tricaine was added to cover the immobilized larvae. Confocal microscopy was performed at 26 ℃. A Leica SP8 (Leica Microsystems, Heerbrugg, Switzerland) automated upright confocal laser scanning microscope allowing simultaneous acquisition of three fluorescent channels and one bright field or differential interference contrast (DIC) was used. The detection system in this microscope is equipped with two photomultiplier tubes and a hybrid detector and a 20×water immersion objective (HC PL APO NA-0.5 WD-3.5 mm) was used to image live infected larvae. The 4D images produced by the

time-lapse acquisitions were processed, clipped, examined and interpreted using the Imaris software. Maximum intensity projection was used to project developed Z-stacks and files were exported in AVI format. To mount figures, frames captured from AVI files were handled using Photoshop software. Imaris software was used to crop and annotate the exported AVI files, then compressed and converted into QuickTime movies with QuickTime Pro software.

RESULTS

Infection of zebrafish larvae with *Cronobacter* via microinjection is lethal

To develop a *Cronobacter turicensis* infection model in a genetically tractable vertebrate model host, we investigated whether the strain *C. turicensis* LMG 23827^T, a strain originally isolated from a fatal neonatal infection could lethally infect zebrafish embryos at 2 dpf. Initial experiments to infect zebrafish with this strain by immersing dechorionated embryos in a suspension of strain LMG 23827^T failed. Lethality required high concentrations of strain LMG 23827^T or LMG 23827^T::GFP (10^9–10^{10} CFUs/mL), and the experiments were not reproducible (data not shown). Similar results were reported for co-infection experiments using other pathogens under static immersion conditions.[34] Moreover, using the bath immersion experimental design, we were unable to establish a stable infection in the digestive system of the larvae. Similar observations were reported in the study by Levraud *et al.*[23] showing that zebrafish embryos were not susceptible to oral infection with *Listeria monocytogenes*.

Therefore, we next focused on the possibility of introducing LMG 23827^T or LMG 23827^T::GFP into 2 dpf-old embryos directly by microinjection into the yolk sac, the common cardinal vein or the hindbrain ventricle, thereby exploiting the advantage of easy fluorescent tracking by using the GFP transgenic strain *C. turicensis* LMG 23827^T::GFP with concentrations ranging from 50 to 10^4 CFUs. Yolk injections were performed into the posterior part of the yolk sac before the extension to prevent perforation of the common cardinal vein, which widely covers the anterior part of the yolk. After injection, the embryos were transferred into 24-well plates containing fresh E3 medium and further incubated at 28 ℃. Signs of disease, larval survival, fluorescence pattern of bacteria, and bacterial load were examined over time. The bacterial load was determined by counting the CFU of homogenates of whole individual larva plated on agar plates containing kanamycin for selection purposes at 37 ℃ overnight.

Intravenous injections of up to 10^4 CFUs and hindbrain injections of up to 10^3 CFUs did not result in an apparent infection. Bacteria were cleared from the system within the first 24 hpi, and the larvae showed no indication of disease or mortality (data not shown). However, injections of as little as 50 CFUs into the yolk resulted in the rapid replication of *Cronobacter* inside the yolk sac of approximately two log units within 24 hpi and a subsequent spreading into the larval blood stream between 24 and 48 hpi (Figure 1A). Confocal imaging revealed bacteria accumulating at the surface of the yolk sac before further spreading into the larvae (Figure 1B). The bacteria inside the yolk could be found dividing by binary fission (Figures 1C and 1D). The

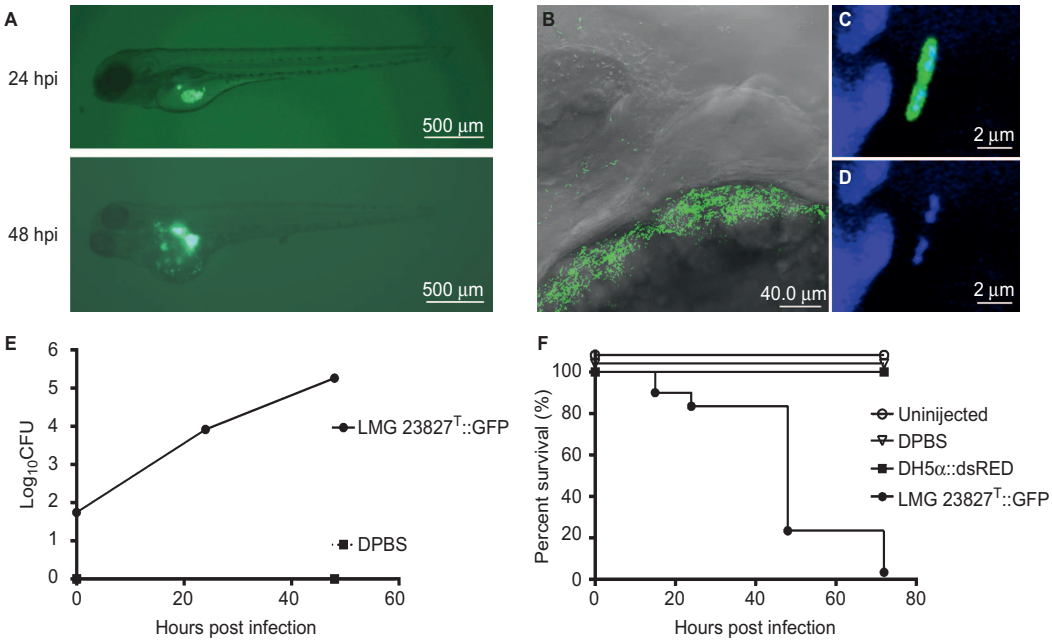

Figure 1 Injection of *C. turicensis* into the yolk of 2-dpf embryos causes lethal infection. (**A**) Appearance of larvae at 24 and 48 hpi under a fluorescence light microscope after injection of 50 CFU of *C. turicensis* LMG 23827^T::GFP into the yolk sac. At 24 hpi, replication of GFP-expressing bacteria and further spreading into the yolk are visible. At 48 hpi, continuous replication and spreading inside the whole yolk sac and also into further tissues of the larvae can be observed. (**B**) CLSM-acquired 3D stack showing part of the border between the yolk sac and the larva in a region close to the head. GFP-expressing bacteria accumulate on the surface of the yolk. Some have already crossed the barrier, distributing in the larva. (**C** and **D**) Inside the yolk, many dividing bacteria can be observed, confirmed by DAPI staining of bacterial and host DNA. The images show merged channels for DIC/GFP (**B**), DAPI/GFP (**C**) or DAPI alone (**D**). (**E**) Mean growth curve of *C. turicensis* inside infected larvae with a starting inoculum of approximately 50 CFU. Enumeration was performed by plating homogenates of whole individual larvae at different time points on selective agar plates at 37 ℃ and subsequent counting of bacterial colonies. (**F**) Survival rates of larvae injected with 50 CFU *C. turicensis* LMG 23827^T::GFP or 50 CFU *E. coli* DH5α::dsRED or 1 nL of DPBS or left uninjected, following incubation at 28 ℃ for 72 hpi.

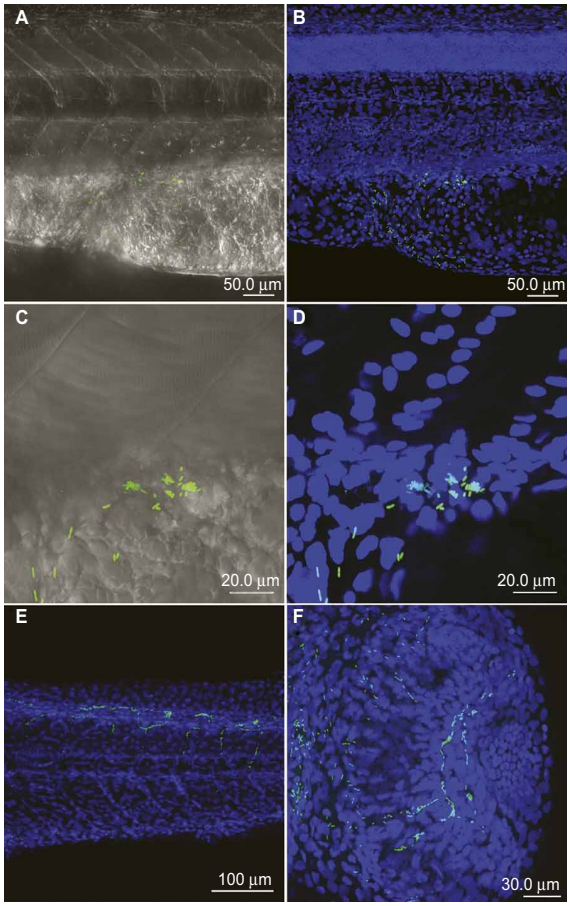

Figure 2 Confocal imaging of DAPI-stained larva after yolk injection of GFP-expressing *Cronobacter* reveals bacteria inside the yolk and the bloodstream of the larva. At 24 hpi, the bacteria have spread from the initial injection site into the extension part of the yolk sac (**A** and **B**). The rod-shaped bacteria are forming clusters and replicate by binary fission near the barrier between yolk and the vasculature of the larva (**C** and **D**). At 48 hpi, numerous bacteria are visible in the blood circulation of the trunk (**E**) and the eye (**F**), forming clusters and accumulating in the capillaries. DIC/GFP (**A** and **C**) and DAPI/GFP (**B, D, E** and **F**) channels are merged, respectively.

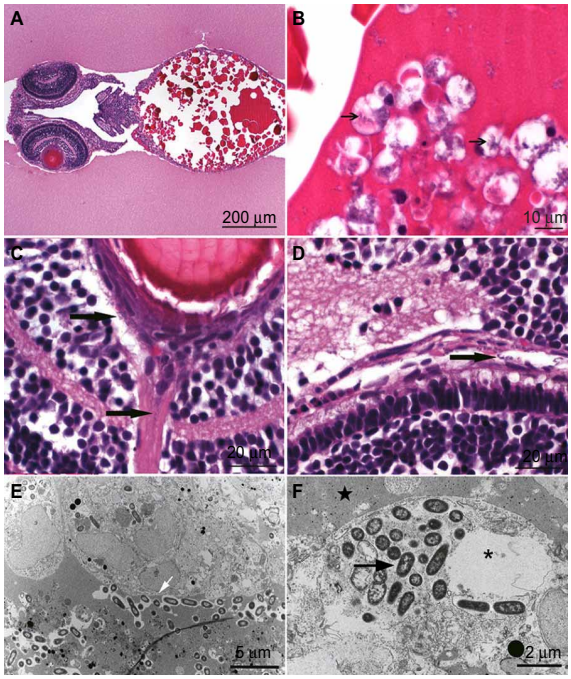

Figure 3 Pathology of the infection. Histological hematoxylin and eosin-stained sections of infected larvae at 48 hpi. (**A**) The overview shows a longitudinal section through the yolk and the head region of a whole larva embedded in egg white. (**B**) Under higher magnification, clusters and single bacteria are visible inside the yolk (black arrows). (**C** and **D**) Further bacteria can be observed in the blood circulation, accumulating in capillaries of the eyes and brain (black arrows). (**E**) TEM imaging of the barrier region between yolk and larva shows bacteria distributed inside but also lining the border of the yolk (white arrow). (**F**) TEM imaging of bacteria (arrow) phagocytosed by an innate immune cell embedded in the yolk (star). The cell is degrading, as indicated by the swelling and fragmentation of their organelles (asterisk).

bacterial load continued to increase for another log unit at the same time (Figure 1E). Mortality after yolk injections increased up to 100 % at 72 hpi, while microinjection of equal or greater numbers of *E. coli* DH5α::dsRED resulted in complete survival of the infected larvae (Figure 1F).

The traverse of *Cronobacter* to the bloodstream could be observed throughout the entire length of the boundary between yolk and vasculature (Figures 2A-2D), followed by an accumulation of bacterial clusters in the capillaries of the trunk and the eyes (Figures 2E and 2F).

Infection progression and pathology

Larvae that were injected into the yolk sac showed at 30-48 hpi small 1×2 µm, rod-shaped bacteria free and intracytoplasmic in leukocytes as macrophages or neutrophils in the yolk sac (Figures 3A and 3B) and in the lumina of several blood vessels, especially close to the eyes and brain (Figures 3C and 3D). The number of macrophages and neutrophils within the yolk sac lining the wall was increased compared with control animals (data not shown).

TEM images showed long rods of 2-µm length and 1-µm width that were lying free in the protein of the yolk sac (Figure 3E). The bacteria contained a thin cell wall, typical for gram-negative rods, and a loose chromatin pattern. Some bacteria were dividing, which could also be observed by confocal microscopy. Some leukocytes containing bacteria showed degeneration as large pale intracytoplasmic vacuolation, crystolysis and swelling of mitochondria, increase of lipid globules or dilation or even fragmentation of Golgi or endoplasmic reticulum (Figure 3F). Additional signs of degeneration, such as karyorhexis, karyopyknosis and hypereosinophilia of the cytoplasm, were observed in many leukocytes (Figure 3F). The presence of neutrophils inside the yolk sack was confirmed by confocal imaging, where labeled neutrophils were associated with the bacteria (Figure 4).

Innate immune response to *Cronobacter* infection (CLSM Leica SP8 – live imaging)

To visualize the dynamics of *Cronobacter* replication and the innate immune reaction to the infection inside the yolk, we utilized transgenic 2 dpf zebrafish embryos of the *Tg(lyz:DsRED2)nz50* line, which harbor red fluorescent protein-expressing neutrophils. These embryos were injected with a dose of approximately 50 CFUs of GFP-expressing

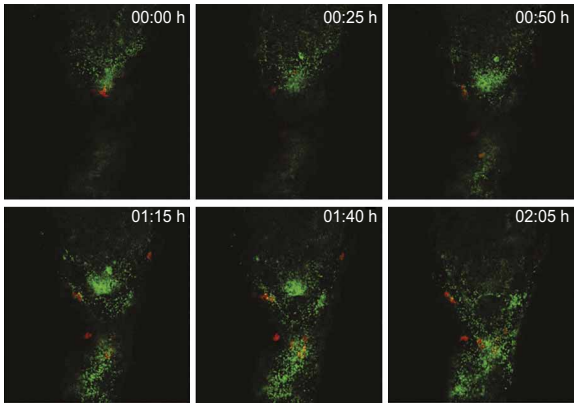

Figure 4 *C. turicensis* rapidly replicates inside the yolk and initiates an innate immune response with recruitment of neutrophils (magnification 10×). Live imaging of the replication of *C. turicensis* LMG 23827^T::GFP after injection of 50 CFU into the yolk of 2-dpf transgenic *Tg(lyz:DsRED2)nz50* zebrafish embryos, possessing red fluorescent neutrophils. Rapid replication and clustering of the bacteria can be observed, which readily induced recruitment of neutrophils into the yolk sac. Live imaging was performed using a CLSM Leica SP8 over a time course of approximately 2 h.

C. turicensis LMG 23827^T::GFP into the yolk sac at 2 dpf. The *Cronobacter*-host interactions were captured in real-time at 24 hpi using high-resolution confocal laser scanning microscopy for a time course of approximately 2 h. We observed rapid replication of *Cronobacter* inside the yolk forming several clusters of motile bacteria. Over time, an increasing number of red fluorescent neutrophils was recruited to the yolk taking up the bacteria but were unable to control the proliferation and spreading of *Cronobacter* (Figure 4, Supplementary Video).

Drug screening

For the next step, we determined whether the zebrafish model may be suitable for testing the effectiveness of antimicrobial agents to clear infections with *C. turicensis* LMG 23827^T. Prior to these experiments, the minimum inhibitory concentrations (MICs) of a selection of antimicrobial drugs belonging to different antibiotic classes were determined for the wild type *C. turicensis* LMG 23827^T as well as the GFP-expressing strain *C. turicensis* LMG 23827^T::GFPKan^R using E-test strips (bioMerieux, Marcy-l-Etoile, France) on Müller Hinton agar, according to the recommendations of the manufacturer. The following MIC values (in mg/L) were determined for both strains: ampicillin, 0.75; tetracycline, 1.5; cephalothin, 6; rifampicin, 2; gentamicin, 0.38; polymyxin B, 0.094; nalidixic acid, 0.5; and chloramphenicol, 64. Conversion of the MIC data into qualitative categories using the European Committee on Antimicrobial Susceptibility Testing breakpoints suggested that *C. turicensis* LMG 23827^T was susceptible to all tested antibiotics with exception of rifampicin and chloramphenicol. There was no variation among the wild type and its GFP variant (data not shown).

Based on these findings, we tested the activity of ampicillin, tetracycline and nalidixic acid against *Cronobacter in vivo* in the zebrafish infection model. The embryos were tested for antibiotic toxicity prior to the start of this experiment. Embryos in E3 medium supplemented with tetracycline or ampicillin did not exhibit any toxic effect and/or mortality; however, the embryos exposed to nalidixic acid exhibited pericardial edema but no mortality (data not shown). Because all three antibiotics are water soluble, they were administered to the fish water after the yolk injections of *Cronobacter* or DPBS as control. Another control group was infected but left without treatment. The distribution of *Cronobacter* within the infected larvae was followed by fluorescence microscopy after the injection of the GFP-expressing strain (Figure 5A). The survival rate (Figure 5B) and the bacterial load (Figure 5C) were determined for individual larvae by microscopic observation and plate count enumeration. While treatment with tetracycline had no significant effect on the bacterial load and survival, treatment with ampicillin significantly reduced the bacterial load compared with untreated larvae, but did not increase survival. However, treatment with nalidixic acid had a significant impact on both bacterial load and the survival of infected larvae. At 24 hpi, *Cronobacter* could no longer be detected in these larvae by plate count or by fluorescence microscopy. Furthermore, the survival rate was close to 100 % at 72 hpi. Interestingly, during treatment with nalidixic acid, the formation of pericardial edema was observed in nearly 100% of all larvae.

FkpA is an important virulence factor for *Cronobacter turicensis* infection in zebrafish embryos

Given the previous results and observations concerning the behavior of *Cronobacter* and innate immune cells after injection into the yolk sac, we concluded that internalization and survival of *Cronobacter* cells in professional phagocytes of the innate immune system, such as macrophages present in the yolk and/or the blood stream, play a key role during the infection process. In a recent study, the eminent role of a functional FkpA (also known as macrophage infectivity potentiator-like protein) in survival and replication in human macrophages was reported for *C. turicensis* LMG 23827^T.[28] We therefore tested a Δ*fkpA* in-frame mutant for attenuated pathogenicity in infection experiments using the above-described experimental design; *C. turicensis* LMG 23827^T::dsRED served as control. The survival rate at 48 hpi increased to approximately 70 % for ΔfkpA::dsRED mutant-injected larvae (Figure 6A), thus confirming the role of this gene as a pathogenicity factor during *Cronobacter* infection in zebrafish embryos. Furthermore, the bacterial load in *ΔfkpA*::dsRED mutant (*C. turicensis* LMG 23827^T *ΔfkpA*::dsRED)-injected larvae was significantly lower compared with the control group (Figures 6B and 6C). Injection experiments using the complemented mutant strain (*C. turicensis* LMG 23827^T Δ*fkpA*::*fkpA*) resulted in a lower survival rate and a higher bacterial load, whereas control experiments using the mutant strain transformed with the vector alone (*C. turicensis* LMG 23827^T Δ*fkpA*::pCCR9) yielded survival rates and bacterial loads comparable to the ones observed in the Δ*fkpA*::dsRED mutant-injection experiments (Figures 6A-6C).

DISCUSSION

To adopt the zebrafish model to study these human opportunistic pathogens, several experimental designs were used. As infecting the embryos via the oral route proved unsuccessful, we focused on introducing the pathogens into the animal circulation system via microinjection. However, injection of up to 10 000 CFUs into the cardinal vein of 2 dpf old zebrafish larvae did not result in clinical signs of infection in the embryos. Fluorescence microscopy showed that no replication occurred and the bacteria were cleared instead within 24 hpi (data not shown). In fact, it has been reported that *Cronobacter* spp. exhibit only a "moderate" capability to survive in human blood or serum. Thus, it was shown for *C. sakazakii,* which is closely related to *C. turicensis*, that a minimum of a two-log reduction of a bacterial inoculum occurred within a 30-min exposure in 50% human blood.[35]

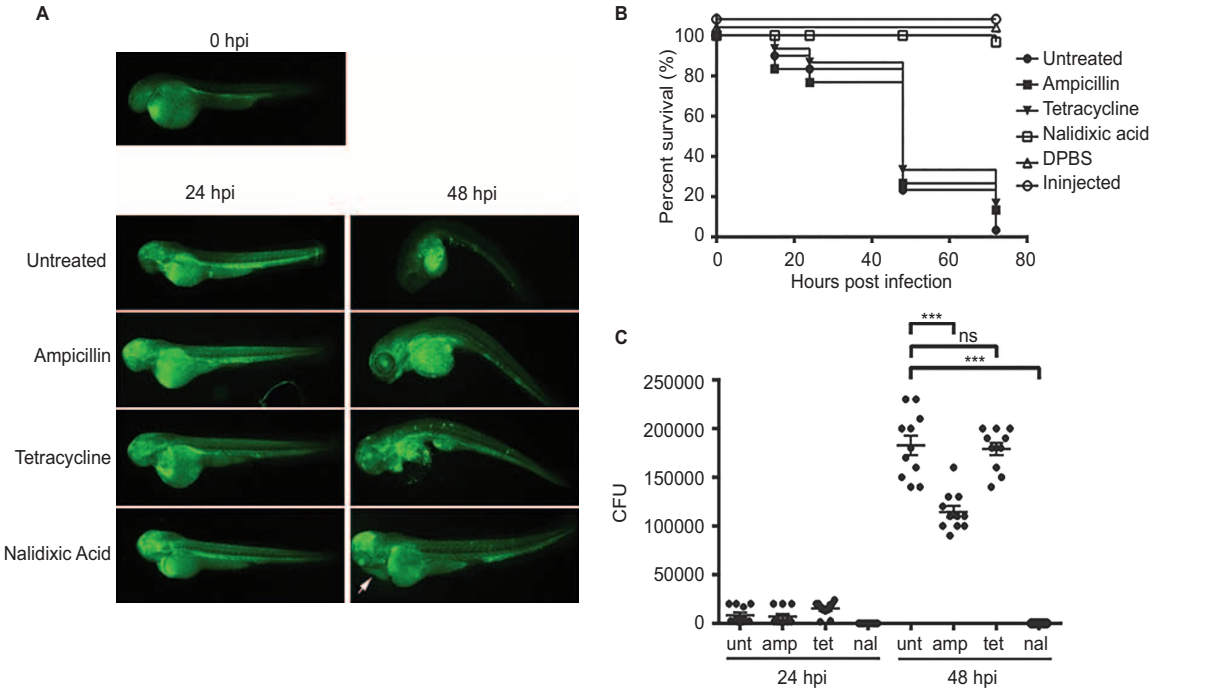

Figure 5 Effects of antibiotic drugs on an infection of ***C. turicensis*** in zebrafish larvae. (**A**) Fluorescence light microscopic appearance of representative larvae at different time points after the injection of ***C. turicensis*** LMG 23827^T::GFP into the yolk sac at 2 dpf and subsequent treatment with 8 mg/mL ampicillin, 8 mg/mL tetracycline or 4 mg/mL nalidixic acid or without treatment (magnification 40×). Larvae treated with nalidixic acid exhibited the formation of pericardial edema at 48 hpi (white arrow) (**B**) Survival rates of infected larvae with and without treatment with various antibiotics and of control larvae injected with DPBS or uninjected controls are shown. (**C**) Quantification of the bacterial load of individual larvae at different time points and under different treatment conditions is depicted. Significant differences could be observed at 48 hpi for the treatment with ampicillin and nalidixic acid compared with untreated larvae. Statistical analysis was performed by one-way ANOVA with Bonferroni's posttest. ***, $P<0.001$; ns, not significant. Mean values ±SEM are shown by horizontal bars.

In a recent study, similar observations were reported for zebrafish larvae infection studies on *Staphylococcus epidermidis* when comparing the results from yolk injection versus blood stream (caudal vein) injection experiments.[36] Within this study, it was speculated that the better survival rate of the bacteria injected in the yolk may be explained by several factors (or a combination of those), such as repeated cycles of invasion from the yolk and/or that internalization and proliferation of bacteria in the yolk results in "priming" to an infectious growth strategy but also alternations of the host immune system due to the prolonged exposure to high numbers of bacteria were suggested.

Thus, we concluded that the yolk injection is a uniquely suitable infection site. We showed that, following injection of as few as 50 CFUs, the bacteria were rapidly replicating both freely in the yolk as well as internalized in the primitive macrophages and neutrophils present in the yolk sac. The affected leucocytes were not killed rapidly, and the phagocytosed bacteria did not escape from these cells. At 24 hpi, free bacteria as well as infected macrophages were lining up near the border of the yolk sac, where a traverse into the blood vessel and the surrounding tissue was observed. At later time points (30 hpi – 48 hpi), bacteria colonized the lumina of small blood vessels, especially those close to the eyes and brain. Accumulation of bacteria in the capillaries of the eyes is a typical feature for septicemia and bacteremia in fish. At these sites, the bacteria were observed to form micro-colonies in which they replicated and from which they were shed into the circulation. Thus, during infection in zebrafish embryos, a combination of extracellular and intracellular replication of *Cronobacter* could be observed.

In the next experiment, a selection of antimicrobial compounds was tested for their ability to cure a *Cronobacter* infection in the embryos by application of the drugs to the fish water. Given that the antibiotic concentrations applied were previously shown to be lethal for the bacteria *in vitro*, we can only speculate why the ampicillin and tetracycline treatments were not effective *in vivo*. One explanation may be that the molecules are not reaching lethal concentrations at the predominant infection site inside the yolk, which could be influenced by the size of the molecules, the local pH or the acid/base character of the antimicrobial substance. Nalidixic acid was the only drug to prove efficient in killing the bacteria inside the host. Although the survival rate of infected fishes was close to 100% after 72 hpi, pericardial edema was observed during drug treatment. Interestingly, similar adverse effects, namely, increased intracranial pressure leading to the formation of cerebral edema, have been reported in humans, especially in infants and young children after treatment with nalidixic acid.[37] This result also underlines that zebrafish larvae can show typical reactions to other influencing factors, such as antibiotic treatment, and can thus be used as a drug screening model not only to test the efficacy of a drug against the pathogen *in vivo* but also to predict possible adverse effects on the patient.

It has been shown that *Cronobacter* spp. are capable of surviving and replicating within human macrophages for up to 96 h, and, in a recent study, the FkpA protein was identified as a key factor involved in this

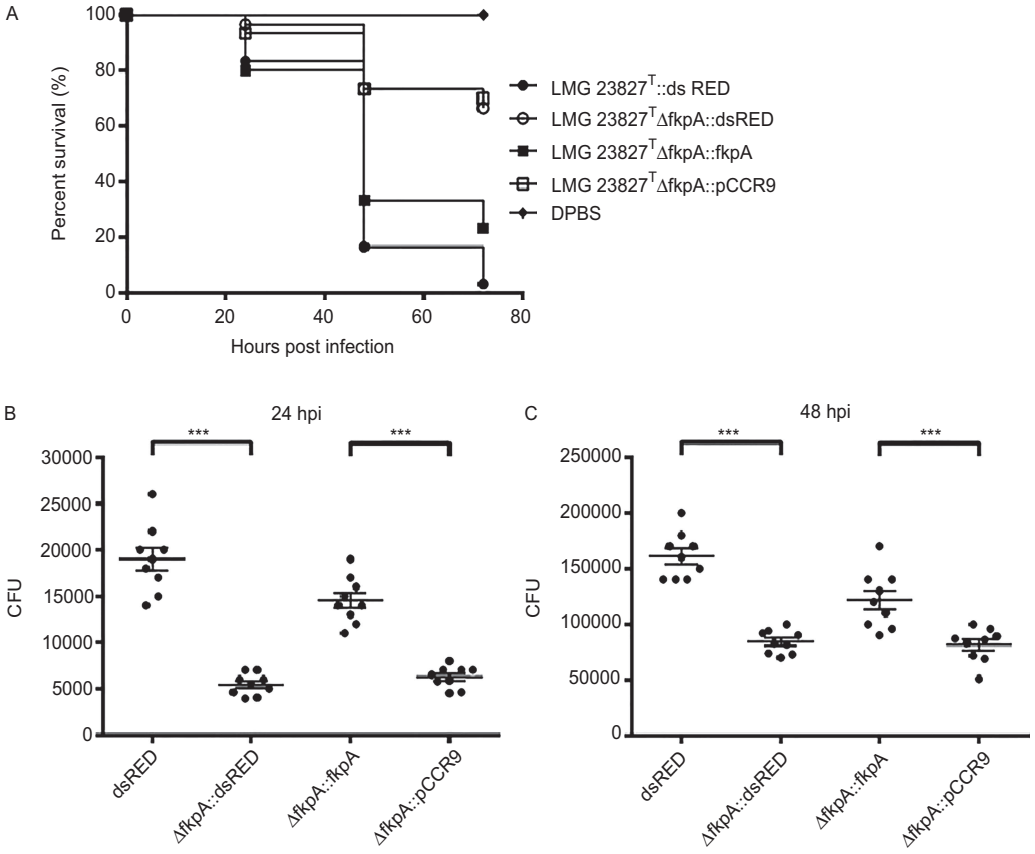

Figure 6 The mutant *C. turicensis* LMG 23827^T Δ*fkpA* shows attenuated pathogenicity in zebrafish larvae. (**A**) Survival rates of larvae injected with 50 CFU of *C. turicensis* LMG 23827^T::dsRED or LMG 23827^T Δ*fkpA* ::dsRED or LMG 23827^T Δ*fkpA::fkpA* or LMG 23827^T Δ*fkpA*::pCCR9 or 1 nL of DPBS alone as negative control at 2 dpf into the yolk are shown. (**B** and **C**) Quantification of the bacterial load of individual larvae at 24 and 48 hpi shows significant differences between wild type and mutant bacteria. Statistical analysis was performed by one-way ANOVA with Bonferroni's posttest. ***, $P<0.001$. Mean values ± SEM are shown by horizontal bars.

ability. When using an *fkpA* mutant that is defective in survival and replication in human macrophages, pathogenicity was strongly attenuated in the zebrafish model. This finding provides evidence that macrophages and other phagocytic cells can play a crucial role in the process of traversing physical barriers such as epithelia and endothelia and, by this, promote further systemic spreading of intracellular bacterial pathogens within the host organism.

Taken together, by exploiting the favorable features of the zebrafish within this study, we have established and applied the first zebrafish model to study the pathogenesis of *C. turicensis in vivo*. Our model provides interesting insights into the pathogenic nature of these opportunistic facultative intracellular pathogens and also shows the reaction of the innate immune system to an infection in real time. With the experimental design developed in this study, the results can be obtained within days, and the number of experiments can be easily scaled up. Thus, large numbers of bacterial mutants and strains can be screened for virulence-related factors of *Cronobacter*, providing a promising tool to discover further detailed features of *Cronobacter* virulence and the counteracting innate immune response in future studies.

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Chapter 4

The DSF type quorum sensing signaling system RpfF/R regulates diverse phenotypes in the opportunistic pathogen *Cronobacter*

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Planning of zebrafish experiments, microinjection, bacterial enumeration by plate counting, statistical analysis, preparation of graphs and writing the manuscript.

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The DSF type quorum sensing signalling system RpfF/R regulates diverse phenotypes in the opportunistic pathogen *Cronobacter*

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Several bacterial pathogens produce diffusible signal factor (DSF)-type quorum sensing (QS) signals to control biofilm formation and virulence. Previous work showed that in *Burkholderia cenocepacia* the $RpfF_{Bc}$/RpfR system is involved in sensing and responding to DSF signals and that this signal/sensor gene pair is highly conserved in several bacterial species including *Cronobacter* spp. Here we show that *C. turicensis* LMG 23827^T possesses a functional RpfF/R system that is involved in the regulation of various phenotypes, including colony morphology, biofilm formation and swarming motility. *In vivo* experiments using the zebrafish embryo model revealed a role of this regulatory system in virulence of this opportunistic pathogen. We provide evidence that the RpfF/R system modulates the intracellular c-di-GMP level of the organism, an effect that may underpin the alteration in phenotype and thus the regulated phenotypes may be a consequence thereof. This first report on an RpfF/R-type QS system of an organism outside the genus *Burkholderia* revealed that both the underlying molecular mechanisms as well as the regulated functions show a high degree of conservation.

Members of the genus *Cronobacter* spp. are considered opportunistic pathogens associated with rare but severe neonatal systemic infections predominantly in pre-term and/or low birth weight infants and thus have attracted the attention of public health authorities and researchers in the past[1–3].

Epidemiological investigation of outbreaks of *Cronobacter* spp. infections in hospitals indicated powdered infant formula as a source of contamination, when these organisms were isolated from both reconstituted milk as well as from milk feeding equipment and utensils. The latter may be enhanced by the organism's ability to adhere and form biofilms on many surfaces, including silicone, latex, polycarbonate (used in the feeding bottle manufacture) and stainless steel[4,5].

Bacterial processes involved in biofilm formation and virulence, are often controled by quorum sensing (QS), a mechanism based on the production, release and detection of signaling molecules of low molar mass. Extracellular concentrations of signal molecules are sensed by the bacteria and, upon reaching a population density-dependent threshold, they are detected by the cells, which in turn induce target gene expression in a coordinated fashion[6–9].

To date many structurally unrelated signal molecules have been identified, including *N*-acyl-homoserine lactones (AHLs) in Gram-negative bacteria, oligopeptides in many Gram-positive bacteria and autoinducer-2 (AI-2), which is thought to serve as a signal for interspecies communication[7–9].

Another group of signal molecules are the *cis*-2-unsaturated fatty acids, often referred to as DSF (diffusible signal factor) family signals[10]. The first fatty acid signal, *cis*-11-methyl-2-dodecenoic acid, was identified in the culture supernatant of the phytopathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*)[11]. Subsequently, fatty

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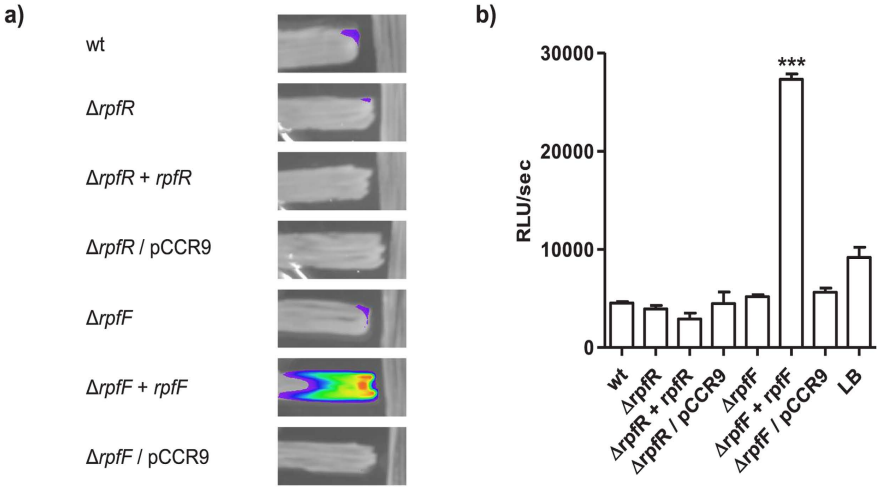

Figure 1. Overexpression of *rpfF* activates the biosensor *B. cenocepacia* H111 –rpfF$_{Bc}$/pAN-L15, which is capable of detecting various DSF family signals. (**a**) The *C. turicensis* LMG 23827^{T} wild type (wt), the mutants (Δ*rpfF,* Δ*rpfR),* the complemented mutants (Δ*rpfF* + *rpfF,* Δ*rpfR* + *rpfR*) and the mutants carrying the empty vector (Δ*rpfF*/pCCR9, Δ*rpfR*/pCCR9) (vertical) were tested in cross-streak experiments against the biosensor (horizontal). The biosensor was clearly induced by the complemented *rpfF* mutant (Δ*rpfF* + *rpfF).* (**b**) The strains were also tested for the production of DSF family molecules in liquid assays. As with the cross streaking, induction of the biosensor was only observed with the Δ*rpfF* + *rpfF* strain. Error bars indicate SEM, n = 4; *P < 0.05 (ANOVA, oneway).

acid-based QS-systems were also identified and in members of the genera *Xylella* and *Stenotrophomonas*[12,13] where they were shown to control the production of virulence factors[11]. More recent work showed that *Burkholderia cenocepacia* produces the signal molecule *cis*-2-dodecenoic acid, which was named BDSF (*B*urkholderia *d*iffusible *s*ignal *f*actor)[14]. BDSF is synthesized by the enoyl-CoA hydratase RpfF$_{Bc}$[15] and is sensed by the receptor protein RpfR, which contains PAS-GGDEF-EAL domains[16]. Binding of BDSF to the PAS domain stimulates the c-di-GMP phosphodiesterase activity of RpfR, which in turn lowers the intracellular c-di GMP level. This signal transduction relay is very different from the one originally described for *X. campestris*, in which the DSF receptor RpfC is a hybrid sensor kinase that phosphorylates its cognate response regulator RpfG. This regulator contains in addition to a REC domain a HD-GYP domain, which is responsible for the c-di-GMP phosphodiesterase activity of the protein[17].

Interestingly, homologs of RpfF$_{Bc}$ and RpfR are present not only in many *Burkholderia* species but also in strains belonging to the genera *Achromobacter*, *Yersinia*, *Serratia*, *Enterobacter* and *Cronobacter*[16], suggesting that RpfF/R type signaling systems may by far more widespread than anticipated. In this study we analysed the RpfF/R system of the clinical strain *Cronobacter turicensis* LMG 23827^{T}, and show that it is involved in the regulation of biofilm formation, macrocolony morphology, proteolytic activity and virulence.

Results and Discussion

RpfF directs the synthesis of a DSF family signal molecule and negatively regulates intracellular c-di-GMP levels in C. turicensis. Previous work identified homologs of both RpfR and RpfF from *B. cenocepacia* in *C. turicensis* LMG 23827^{T}[16]. To investigate the role of this putative QS system in this organism we constructed defined mutants as well as genetically complemented derivatives thereof. We tested the strains for the production of DSF family signal molecules by the aid of the *Burkholderia*-based biosensor H111–rpfF$_{Bc}$ pAN-L15 both in cross-streaking and liquid culture experiments. Under the conditions tested the wild type strain did not induce the biosensor. However, the complemented *rpfF* mutant, in which the wild type allele is expressed from a plasmid, clearly induced the biosensor (Fig. 1), suggesting that RpfF directs the biosynthesis of a *cis*-2 fatty acid signal molecule. We hypothesize that under standard laboratory conditions the amount of signal released by the wild type strain is below the detection limit of our bioassay but that the complemented strain, in which *rpfF* is expressed from a plasmid, produces sufficiently high amounts to induce the biosensor.

RpfR family proteins contain a GGDEF as well as an EAL domain which are associated with the synthesis and degradation of c-di-GMP respectively[18]. The *C. turicensis* LMG 23827^{T} RpfR homolog CBA31265 exhibits an identical domain structure. In order to evaluate the role of RpfR in this strain the intracellular c-di-GMP levels were determined in the wild type, the *rpfR* and *rpfF* mutants and in the complemented strains Δ*rpfR* + *rpfR* and Δ*rpfF* + *rpfF*. The intracellular c-di-GMP level of the *rpfR* and *rpfF* mutants was found to be 3.9-fold or 3.4-fold increased relative to the wild type. Genetic complementation of the mutant reduced the c-di-GMP level to the level of the wild type. These results suggest that both RpfR and RpfF have a negative effect on the intracellular c-di-GMP level (Fig. 2). This is in agreement with the finding that RpfR in *B. cenocepacia* exhibits a net phosphodiesterase activity[16].

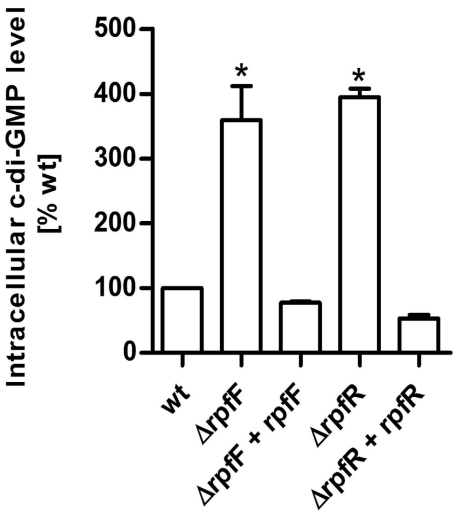

Figure 2. RpfR and RpfF affect the intracellular c-di-GMP level. The intracellular c-di-GMP level was significantly increased in the *rpfR* and *rpfF* mutants relative to the wild type. Detection was performed by by LC-MS/MS. Error bars indicate SEM, n = 2; *P < 0.05 (ANOVA, oneway).

RpfF/R plays a role in quorum sensing regulated phenotypes in C. turicensis LMG 23827^T. We next investigated whether the RpfF/R system is involved in the regulation of typically QS-associated phenotypes. In contrast to the wild type we observed a rough colony morphology of the *rpfR* and to a lesser degree with the *rpfF* mutant on Congo red agar plates (Fig. 3). The strong pinkish colour of the *rpfR* mutant relative to the wild type may suggest an increased production of cellulose and/or curli[19]. In order to support this hypothesis we performed expression studies targeting the gene coding for the catalytic subunit of the cellulose synthase *bscA* as well as the major curli subunit *csgA*. Expression of both genes was considerably increased in the mutants compared to the wild type. However, complementation only partially restored their expression (Fig. 4).

In addition, we observed that both mutants showed reduced proteolytic activity (Fig. 3). Swarming motility on NYG +0.4% agar was not significantly affected by inactivation of the RpfF/R system. However, the complemented mutants exhibited increased swarming motility (Fig. 5). These results are supported by the results of the RT qPCR experiments targeting the flagellar regulon-associated gene *flhE*, which was unaltered in the *rpfF/R* mutants but significantly higher in the complemented mutants (Fig. 4). This finding may be explained by a dose effect due to the additional copies of this gene in the complemented mutants.

Both mutants formed significantly more biofilm under static conditions in microtiter plates (Fig. 6a) than the parental strain. Complementation of the mutants partially restored the wild type phenotype. In the study by Hartmann *et al.* (2010)[20] genes involved in biofilm formation in the closely related species *Cronobacter sakazakii* were identified using a transposon mutagenesis approach. *BscA* and *flhE* were – amongst others – two of the genes that were found to contribute to biofilm formation. Our expression analysis performed in this study suggests that *bcsA* but not *flhE* is regulated by the rpfF/R system (Fig. 4).

Importantly, the strains Δ*rpfR* + *rpfR* and Δ*rpfF*/pCCR9 showed growth defects and did not reach the same OD as the other strains, which may explain the poor complementation of the Δ*rpfR* mutant. The growth curves of wild type strains, complemented mutants and mutants carrying the pCCR9 vector are depicted in Supplementary Figure S1. Partial restoration was also observed when the *rpfF* mutant was supplemented with at least 1 μM BDSF or 20 μM DSF (Fig. 6b). We also tested the various strains for pellicle formation, i.e. biofilm formation at the liquid-air interface. Both mutants showed increased pellicle formation and complementation restored the wild type behavior (Fig. 6c). In a study by Lehner *et al.* (2005)[21] it has been reported that cellulose is one of the major components present in pellicles formed in *Cronobacter* spp. strains. The increased expression levels of *bcsA* in the mutants as observed in our study suggest a negative influence of the RpfF/R regulon in *C. turicensis* biofilm formation. This is in contrast to the homologous system of *B. cenocepacia*[16] but similar to the genetically different DSF-dependent RpfCG systems of *Stenotrophomonas maltophilia* E77 or *X. campestris* pv. *campestris*[22,23].

Zebrafish infection studies. We tested the Δ*rpfF* and the Δ*rpfR* mutants for pathogenicity in a zebrafish infection model. The dsRed-labeled wild type strain *C. turicensis* LMG23827^T (wt::dsRed) served as control. The mortality rate of the zebrafish larvae at 48 hpi increased to approximately 90% for injection with wt::dsRed whereas the mortality rate decreased to 50% when the larvae were infected with the mutant Δ*rpfF* (Fig. 7a). Furthermore, the bacterial load was significantly lower with the *rpfF* mutant when compared with the wild type control (Fig. 7b) indicating a role of the RpfF/R system in the expression of virulence factors required for pathogenicity in the zebrafish model. Injection experiments using the complemented mutant strain (Δ*rpfF* + *rpfF*) resulted in higher mortality rate and higher bacterial load, whereas control experiments using the mutant strain

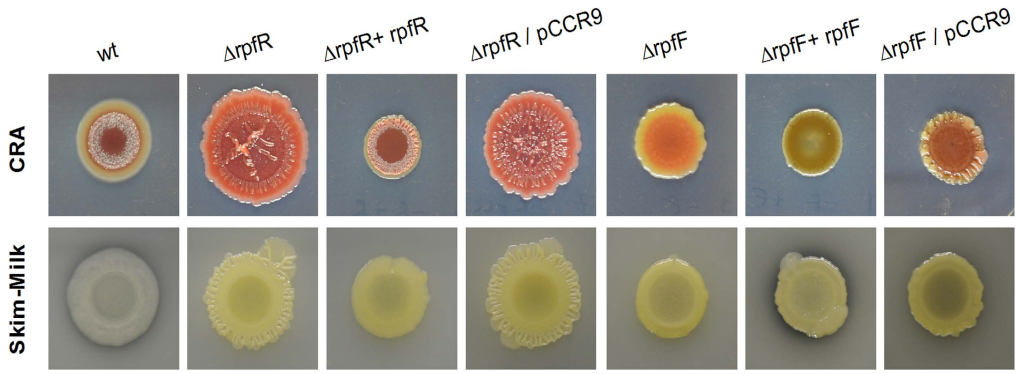

Figure 3. The RpfF/R QS-system controls colony morphology and protease production. Deletion of *rpfR* induced a rough, wrinkly colony morphology and increased EPS production on Congo red agar plates (CRA, upper panel). Both the Δ*rpfR* and the Δ*rpfF* mutant showed reduced protease production on skim-milk plates compared to the wild type (lower panel).

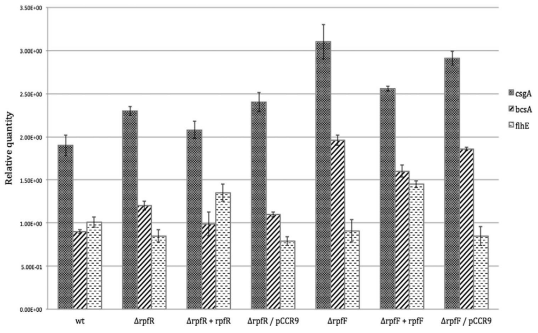

Figure 4. RT-qPCR analysis of *csgA*, *bcsA* and *flhE* gene expression in *C. turicensis* LMG 23827^T (wt), the mutants (Δ*rpfF*, Δ*rpfR*), the complemented mutants (Δ*rpfF* + *rpfF*, Δ*rpfR* + *rpfR*) and the mutants carrying the empty vector (Δ*rpfF*/pCCR9, Δ*rpfR*/pCCR9). The respective mRNA levels were normalized to the 16S rRNA reference gene. Error bars indicate SEM, n = 3.

transformed with the vector alone (Δ*rpfF*/pCCR9) yielded mortality rates and bacterial loads comparable to the ones observed in the Δ*rpfF* mutant experiments (data not shown).

Intriguingly, the mortality rate of larvae injected with the Δ*rpfR* strain was virtually indistinguishable from the wild type.

Here we have shown that *C. turicensis* posesses a RpfF/R family QS system which relies on a *cis*-2-unsaturated fatty acid signal molecule. RpfF/R-type QS systems are particularly widespread among members of the genus *Burkholderia*[10]. We analysed for the first time a RpfF/R family QS system in a bacterium not belonging to the genus *Burkholderia* and demonstrated that despite the phylogenetic distance (β *versus* γ subdivision of proteobacteria) both the molecular mechanism as well as the regulated phenotypes are very similar. Like in *B. cenocepacia*, the RpfF/R system was found to affect swarming motility, biofilm formation and virulence in *C. turicensis*. Furthermore, in both organisms the QS system modulates the intracellular secondary messenger c-di-GMP and this in turn appears to regulate the observed QS-dependent phenotypic traits. The finding that the *rpfF* but not the *rpfR* mutant reduced the virulence of *C. turicensis* suggests that an alternative signal receptor may be present in this strain. This is not unprecedented, as in *B. cenocepacia* an alternative BDSF receptor, BCAM0227, has been identified that is used by some strains as a parallel signaling system to control a subset of functions[24]. However, a bioinformatic analysis neither identified a homolog of BCAM0227 nor of *rpfC*, the DSF receptor of *Xcc*[25].

In conclusion, our data provide evidence that RpfF/R-type QS systems are not restricted to *Burkholderia* sp. but may be widespread among Gram-negative bacteria, in which they influence surface colonization and virulence through modulation of the intracellular c-di-GMP levels. It will be of interest to investigate if homologous systems in other bacteria will control the same phenotypes.

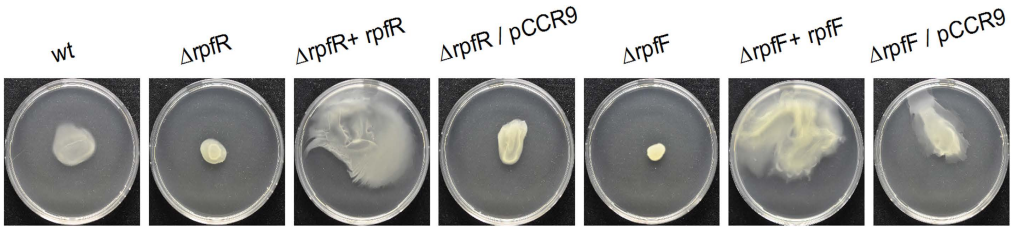

Figure 5. Overexpression of the RpfF/R QS-system increased swarming motility. Strains were spot inoculated on 0.4% NYG agar and plates were photographed after 24 h incubation.

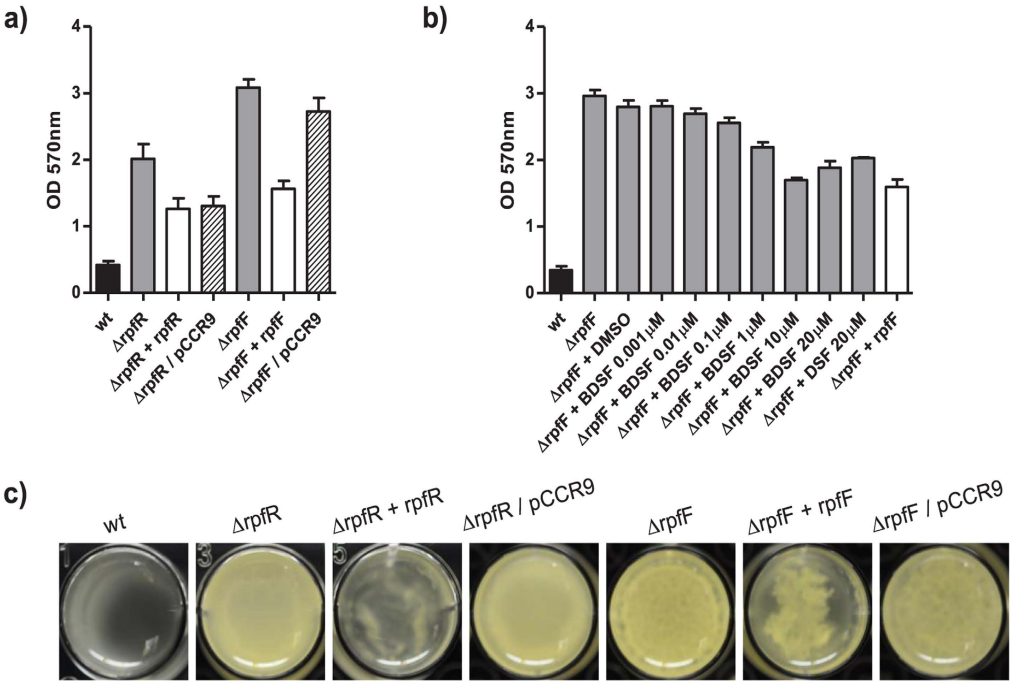

Figure 6. The RpfF/R system regulates biofilm formation under static conditions. (**a**) Deletion of either *rpfR* or *rpfF* increased biofilm formation in microtiter plates. (**b**) Partial restoration was obtained by genetic complementation or by supplementing the medium with BDSF (0.001 μM–20 μM) or DSF (20 μM). Error bars indicate SEM, n > 2. (**c**) Both RpfR and RpfF are involved in pellicle formation tested in NYG broth at room temperature for 48 h.

Material and Methods

Bacterial strains and culture conditions. *C. turicensis* LMG 23827^{T} [26], a clinical isolate responsible for two fatal sepsis cases in neonates in Zurich in 2006 was used in the study. Strains *C. turicensis* LMG 23827^{T}_Nal^{R} as well as *C. turicensis* LMG 23827^{T}/pRZT3::dsRed were described previously[27,28].

For selection purposes, during zebrafish embryo infection experiments, *C. turicensis* LMG 23827^{T}_Δ*rpfF*/pCCR9 as well as *C. turicensis* LMG 23827^{T}_Δ*rpfR*/pCCR9 were constructed by transformation of the strains with the vector using standard methods.

Strains were grown in Luria–Bertani (LB) broth over night at 37 °C with gentle shaking. Where appropriate, culture medium or agar was supplemented with nalidixic acid at 256 mg L^{-1} (*C. turicensis* LMG 23827^{T}_Nal^{R}), chloramphenicol at 30 mg L^{-1} (strains harbouring pDS132) or both (transconjugant strains) or tetracyclin at 50 mg L^{-1} (strains harbouring pCCR9 or pRZT3::dsRed).

For microinjection experiments, the bacteria were harvested by centrifugation at 5000 × *g* for 10 min and washed once in 10 ml of Dubelcco's phosphate buffered saline (DPBS, Life Technologies, Switzerland.) After a second centrifugation step, the cells were resuspended in DPBS, and appropriate dilutions were prepared in DPBS.

DNA extraction and manipulations. Chromosomal DNA was isolated using the DNeasy Blood and Tissue kit, plasmids were extracted with the QIAprep Spin Miniprep or Plasmid Midi kits following the

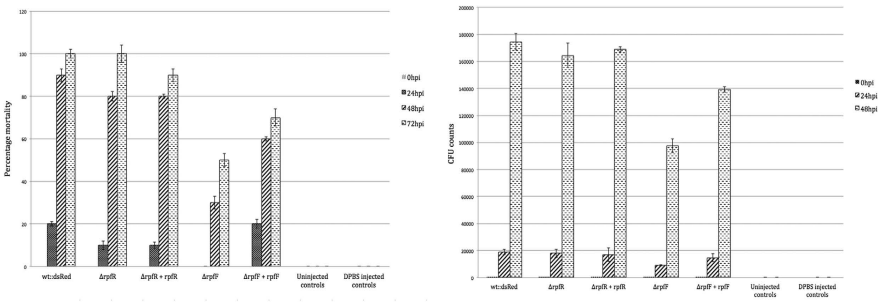

Figure 7. (**a**) Survival rates of zebrafish larvae injected with 50 CFU *C. turicensis* LMG 23827^T/pRZT3::dsRed (wt::dsRed), the mutants Δ*rpfF* and Δ*rpfR* as well as the the complemented mutants Δ*rpfF* + *rpfF* and Δ*rpfR* + *rpfR*. A set of DPBS injected as well uninjected embryos served as controls. Error bars indicate SEM, n = 3. (**b**) Mean growth curve of *C. turicensis* LMG 23827^T/pRZT3::dsRED (wt::dsRed), the mutants Δ*rpfF* and Δ*rpfR* as well as the complemented mutants (Δ*rpfF* + *rpfF,* Δ*rpfR* + *rpfR)* inside infected zebrafish larvae with a starting inoculum of approx. 50 CFU. A set of DPBS injected as well uninjected embryos served as controls. Error bars indicate SEM, n = 3

manufacturer's instructions. For purification purposes (PCR, restriction digest, agarose gel purification) the Qiagen MinElute PCR Cleanup kit or MinElute Gel Purification kit was employed. Enzymes and respective buffers were obtained from Roche Molecula Diagnostics (Rotkreuz, Switzerland) and used according to the manufacturer's instructions.

Construction of C. turicensis LMG 23827^T in frame deletion mutants. Bacterial strains, plasmids and primers used for the construction of mutants are listed in Supplemental Table S1. Deletion mutants of *C. turicensis* LMG 23827^T *rpfF* (CTU_23310) and *rpfR* (CTU_23300) genes were constructed following the protocol described by Philippe *et al.* (2004)[29]. Details are provided in the supplementary material.

Phenotypic assays. Colony morphology: Overnight cultures grown in LB were adjusted to an $OD_{600} = 1.0$ in AB minimal medium[30]. 5 μl of this cell suspension was spottet on CRA plates (2 g Casamino acids, 0.3 g yeast extract, 80 μl of 1 M $MgSO_4$, 4 g agar, dH_2O ad 200 ml, supplemented with 1.6 ml congo red (0.5% in 50% EtOH), 0.65 ml coomassie blue (0.3% in 50% EtOH). The plates were incubated at room temperature for six days before colonies were photographed.

Protease production: Cells of an overnight culture were resuspended in LB and 5 μl cell suspension was spottet on skim milk plates (1% LB agar, 2% w/v skim milk powder). Plates were incubated at 37 °C for two nights and then kept at room temperature.

Swarming motility: Analysis was performed as previously described by Deng *et al.* (2012)[16], except motility was monitored on NYG plates containing 0.5% peptone, 0.3% yeast extract, 2% glycerol and 0.4% agar.

Biofilm formation: Overnight cultures were washed and diluted to an $OD_{600} = 0.01$ in AB minimal medium supplemented with 0.4% glucose and 0.5% casamino acids[30]. 100 μl samples were added to 96 well plates incubated statically for 18 h at 30 °C. Growth was measured at 550 nm in a plate reader (Synergy HT; Bio-Tek, Germany). Surface attached cells were stained by the addition of 100 μl of 1% crystal violet for 30 min at room temperature. The plate was washed thoroughly with tap water and air-dried. To solubilize the stain, 120 μl DMSO was added to each well, incubated for 20 min at room temperature and OD at 570 nm was measured. Data are based on at least 2 independent experiments with 7 technical replicates each.

Bioassays for the production of *cis*-2 fatty acids by using the biosensor *B. cenocepacia* H111 –$rpfF_{Bc}$/pAN-L15. This sensor is sensitive to nM levels of synthetic BDSF and is suitable to detect a wide range of *cis*-2 fatty acid molecules (Suppiger *et al.* submitted). In cross-streaking experiments both the test- and the sensor strain were streaked on LB agar plates close to each other to form a T. The plates were incubated overnight at 37 °C. Following the addition of 10 μl decanal to the lid of the plate the bioluminescence of the sensor strain was visualized using the NightOWL LB 983 (Berthold Technologies, Zug, Switzerland). In liquid bioassays the biosensor was grown in LB broth containing kanamycin 100 μg ml^{-1} to an OD_{600} of 2.0. Overnight cultures of the strains to be tested were centrifuged at 6000 rpm, 5 min and the supernatant (SN) was centrifuged again. 100 μl of this cell-free SN was mixed with 100 μl sensor and incubated for 20 hours at 30 °C. Relative luminescence units (RLU) were obtained by adding 1–2 μl Decanal (Sigma Aldrich, Buchs, Switzerland) to each well and detection was performed using a plate reader (Synergy HT; Bio-Tek, Germany).

Intracellular cyclic-di-GMP level. Bacterial overnight cultures were subcultured in LB medium and 5 ml were harvested at an $OD_{600} = 2.0$ by centrifugation at 5000 rpm, 4 °C. Nucleotide extraction was performed as described by Spangler *et al.* (2010)[31] with slight modifications: cXMP was omitted and the solvent was evaporated in Speedvac at 60 °C. Quantification was performed by LC-MS/MS[32].

Expression analysis of selected genes by RT-qPCR. The expression levels of the 16S rRNA, *csgA, bcsA,* and *flhE* genes in *Cronobacter turicensis* LMG23827^T wild type and its respective *rpfR* and *rpfF* mutants that were

grown in AB medium supplementd with 0.4% glucose and 0.5% casamino acids at 30 °C to early stationary phase were determined using reverse transcription quantitative-PCR (RT-qPCR). 1.5 ml of the above bacterial suspension was re-suspended in 0.5 ml of the lysis buffer of the RNeasyPlus Mini Kit (Qiagen, Hilden, Germany). The samples were transferred on to the lysing bead matrix in MagNA lyser tubes and mechanically disrupted (1 min at 6500 rpm) using the MagNA Lyser Instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland). RNA was isolated from the bacterial lysates following the RNeasy^{Plus}Mini Kit protocol (Qiagen). Genomic DNA was removed by using a genomic DNA binding column and carrying out an on column DNAse I digestion. RNA was eluted in 50 μl of RNAse-free water, and subsequently quantified and quality controlled using the Nanodrop and BioAnalyzer instruments, respectively. 100 ng of RNA were reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). Residual DNA contamination was ruled out in each RNA sample by including a control in which the RT enzyme was omitted. Quantitative PCR was performed on 2.5 ng cDNA using the SYBR green I kit (Roche Molecular Diagnostics), and primers that are listed in Supplemental Table S2 in the LC480 (Roche Molecular Diagnostics) instrument. Following RT PCR conditions were applied for all four genes: 5 min 95 °C followed by 40 cycles of 95 °C for 10 seconds, 50 °C for 20 seconds, 72 °C for 20 seconds, 78 °C for 1 second. Quantification was performed using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics). The *csgA, bcsA, flhE* mRNA levels were normalized using 16S rRNA as reference gene[27].

Zebrafish infection studies. Zebrafish (*Danio rerio*) strains used in this study were albino lines. Husbandry, breeding and microinjection of approx. 50 CFU of bacteria into the yolk sac of 2 dpf embryos was performed following the procedure described in the study by Fehr *et al.* (2015)[28].

A set of uninjected embryos, incubated in E3 maintenance medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) was included in order to determine the quality of the embryos; embryos injected with DPBS served as controls. Injected embryos were transferred into 24-well plates (1 embryo per well) in 1 ml E3 medium per well, incubated at 28 °C and observed for signs of disease and survival under a Leica M165 C stereomicroscope twice a day. In order to follow the course of infection embryos or larvae were collected at several time points, namely at 0, 24, 48 and 72 h post infection (hpi) and individually treated for bacterial enumeration.

Research was conducted with approval (NO 216/2012) from the Veterinary Office, Public Health Department, Canton of Zurich (Switzerland). The applied methods were carried out following the approved guidelines.

Bacterial enumeration by plate counting. The larvae were transferred to 1.5 ml centrifuge tubes and disintegrated by repeated pipetting and vortexing for 3 min in 1 mL of DPBS supplemented with 1% Triton X- 100 (Sigma-Aldrich, Buchs, Switzerland). Subsequently, serial dilutions of this mixture were plated onto LB plates supplemented with tetracycline 50 mg L^{-1} (strains harboring pCCR9 or pRZT3::dsRed). The plates were incubated up to 48 h at 37 °C.

Survival assay. Embryos were microinjected as mentioned above and maintained individually in 24-well plates in E3 medium at 28 °C. The number of dead larvae was determined at different time points visually based on the absence of a heartbeat.

Statistical analysis. Statistics and graphs were performed using GraphPad Prism 6 (GraphPad Software, San Diego, USA). Experiments were executed at least three times, unless stated otherwise. The CFU counts of individual larva at different time points and under different conditions were verified for significant variances by one-way ANOVA with Bonferroni's post-test.

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Author Contributions

A.S. and A.K.E. designed and conducted experiments along with data analyses and manuscript writing; V.K. performed cyclic-di GMP measurements. L.E., A.L. and R.S. guided the work and evaluated manuscript and results; A.S. and A.K.E. equally contributed this work.

Additional Information

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Chapter 5

Linking genomo- and pathotype: exploiting the zebrafish embryo model to investigate the divergent virulence potential among *Cronobacter* spp.

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Contribution:

Planning of experiments, microinjection experiments, bacterial enumeration by plate counting, statistical analysis, preparation of figures and writing the manuscript.

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RESEARCH ARTICLE

Linking Genomo- and Pathotype: Exploiting the Zebrafish Embryo Model to Investigate the Divergent Virulence Potential among *Cronobacter* spp.

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Abstract

Bacteria belonging to the genus *Cronobacter* have been recognized as causative agents of life-threatening systemic infections primarily in premature, low-birth weight and immune-compromised neonates. Apparently not all *Cronobacter* species are linked to infantile infections and it has been proposed that virulence varies among strains. Whole genome comparisons and *in silico* analysis have proven to be powerful tools in elucidating potential virulence determinants, the presence/absence of which may explain the differential virulence behaviour of strains. However, validation of these factors has in the past been hampered by the availability of a suitable neonatal animal model. In the present study we have used zebrafish embryos to model *Cronobacter* infections *in vivo* using wild type and genetically engineered strains. Our experiments confirmed the role of the RepF1B-like plasmids as "virulence plasmids" in *Cronobacter* and underpinned the importantce of two putative virulence factors—*cpa* and *zpx*—in *in vivo* pathogenesis. We propose that by using this model *in vivo* infection studies are now possible on a large scale level which will boost the understanding on the virulence strategies employed by these pathogens.

Introduction

Cronobacter (C.) spp. are Gram-negative, rod-shaped, non- sporeforming, motile bacteria of the family *Enterobacteriaceae*. The genus *Cronobacter*–as proposed in 2008 –currently consists of seven species according to the "List of prokaryotic names with standing in nomenclature" (http://www.bacterio.net/allnamesac.html, viewed, June 6th 2016) and encompasses organisms that have previously been identified as *Enterobacter sakazakii* [1, 2]. The extension of the genus *Cronobacter* by three non-pathogenic *Enterobacter* species (*E. pulveris*, *E. helveticus* and

E. turicensis), as proposed by Brady et al. [3] was withdrawn as the genus membership was experimentally disproved in the study by Stephan et al. [4].

Cronobacter spp. are regarded as opportunistic pathogens linked with life-threatening infections, particularly in premature (< 37 weeks of gestation stage), low-birth weight (< 2500 g) or immuno-compromised neonates and infants less than 4 weeks of age [5, 6] and its occurrence has been epidemiologically linked to the consumption of reconstituted intrinsically or extrinsically contaminated powdered infant formula (PIF) [7, 8].

Clinical symptoms include necrotising enterocolitis (NEC), bacteremia and meningitis, with case fatality rates ranging between 40 and 80% being reported [9, 10].

Apparently not all *Cronobacter* species are linked to infantile infections and it has been proposed that virulence varies among strains. Thus *C. sakazakii*, *C. malonaticus*, and *C. turicensis* are the primary pathogenic species which cause the majority of severe illnesses neonates [11]. Some studies suggest that *C. malonaticus* may be more of an adult pathogen than *C. sakazakii* or *C. turicensis* are [12]. Other species of *Cronobacter* include *C. universalis*, *C. condimenti*, *C. muytjensii*, and *C. dublinensis*. Except for *C. condimenti*, all other *Cronobacter* species cause a variety of infections in humans [1, 7].

Applying a multilocus sequence typing scheme (MLST) to *C. sakazakii* strains revealed distinct pathovars which are clonal lineages of particular clinical significance, namely clonal comlpex 4 (CC4) that contains multilocus sequence type 4 (ST4), as well as ST12. These are strongly associated with invasive meningitis and NEC cases. In addition, *C. malonaticus* clonal complex 7 was found to be linked with adult infections [7, 13, 14, 15].

Whole genome sequencing efforts and *in silico* analysis revealed a substantial amount of genotypic variation and identified potential determinants encoding for potential virulence determinants, present in some strains but absent in others which may explain that not all *Cronobacter* spp. are equally virulent and cause invasive disease such as bacteremia and meningitis [11, 16, 17]. However, the validation of putative virulence components has been hampered by the availability of suitable neonatal animal models. So far, *in vivo* studies have concentrated on the neonatal rat, mouse or gerbil as model organisms [17,18,19,20]. Although valuable information has been obtained from these studies, the lack of possibilities for a real-time analysis and the need for laborious and invasive sample analysis limits the use of mammalian experimental animals.

Alternatively, the nematode *Caenorhabditis elegans* has been used to study *Cronobacter* virulence factors and host immune responses [21]. Thus, the role of the *Cronobacter sakazakii* lipopolysaccharide (LPS) and the p38 MAPK pathway as a major factor in host immune response against LPS-mediated challenges has been elucidated using this model [22]. However, invertebrates are genetically not closely related to humans and their immune system shows many differences from the human immune system.

Zebrafish (*Danio rerio*) are increasingly used as model to study infections with human pathogens as they offer distinct advantages over mammalian models, such as mice and rats or invertebrate animal models such as the nematode *C. elegans*. Being vertebrates, zebrafish are evolutionarily closer to humans than are nematodes and they are easier to work with and to study than mice, but retain the advantage of a similar immune system [23]. The execution of large-scale infection studies in zebrafish is possible due to their fecundity and small size. In a recent study we adapted the zebrafish embryo model to study *Cronobacter* infections [24]. This model proved especially useful, since in embryos only the innate immune system is displayed which resembles the situation during infection in premature infants and neonates.

In the current study we employed the recently developed zebrafish embryo model to (1) determine the virulence spectrum displayed among species and strains within the *Cronobacter* genus, (2) define the role of the proposed "virulence plasmids"in *Cronobacter* spp. and (3)

confirm the influence of two putative virulence determinants—the plasmid encoded Cpa and the chromosomally encoded Zpx—in *in vivo* pathogenesis.

Material and Methods

Bacterial strains and culture conditions

The description of the wild type strains used in the study is given in Table 1. The two plasmid-cured strains *C. turicensis* LMG 23827^{T}ΔpCTU1 and *C. sakazakii* ATCC BAA-894 ΔpESA3 were constructed in the study by Franco et al. [25]. Those strains together with the *C. sakazakii* strain E899, which is naturally devoid for the pESA3 plasmid were provided by CFSAN, FDA, USA. *Cronobacter* spp. and *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth with shaking (210 rpm) or on LB agar. Antibiotics were added when required at the following concentrations: ampicillin (100 mg L^{-1}), chloramphenicol (30 mg L^{-1} and nalidixic acid (256 mg L^{-1}).

A nalidixic acid resistant strain of *C. sakazakii* ATCC BAA-894(Nal256) was constructed according to the method by Johnson et al. [28]. Strain *C. sakazakii* ATCC BAA-894Δ*cpa* was created during transconjugation experiments and was consequently nalidixic acid resistant as well. Both strains were used during quantitative zebrafish embryo infection experiments. Cultures for experiments were grown in LB supplemented with nalidixic acid (256 mg L^{-1})

For microinjection experiments, the bacteria were grown to stationary phase in LB overnight (approx. 12 hours) at 37°C, harvested by centrifugation at 5000 x *g* for 10 min and washed once in 10 ml of Dubelcco`s phosphate buffered saline (DPBS, Life Technologies.) After a second centrifugation step, the cells were resuspended in DPBS, and appropriate dilutions were prepared in DPBS.

DNA extraction and manipulations

Chromosomal DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen), plasmids were extracted with the QIAprep Spin Miniprep or Plasmid Midi kits (Qiagen) following the

Table 1. Wild type strains used in the zebrafish embryo infection experiments.

Species	Strain ID ATCC[a] LMG[b], other*	O type[c]	Sequence type[d]	Source and/or reference
Cronobacter condimenti	LMG 26250^T	O1	98	Spiced meat [2]
Cronobacter dublinensis subsp. dublinensis	LMG 23823^T	O1b	106	Milk powder processing environment [1]
Cronobacter dublinensis subsp. lactaridi	LMG 23815	O1a	79	Milk powder processing environment [1]
Cronobacter dublinensis subsp. lausannensis	LMG 23824	O2	80	Basin of a water fountain [1]
Cronobacter malonaticus	LMG 23826^T	O2	7	Human breast abscess [1]
Cronobacter muytjensii	ATCC 51329^T	O2	81	Unknown [1]
Cronobacter sakazakii	ATCC 29544^T	O1	8	Child throat [1]
Cronobacter sakazakii	ATCC BAA-894	O1	1	Milk powder [26]
Cronobacter sakazakii	E899*	O2	4	Clinical
Cronobacter turicensis	LMG 23827^T	O1	19	Neonate [1]
Cronobacter universalis	LMG 26249^T	O1	54	Water [1, 2]
Escherichia coli	XI1 blue	n. a.	n.a.	Agilent

[a]: ATCC = American Type Culture Collection, Manassas, USA

[b]: LMG = BCCM/LMG Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium

*: Type culture collection Institute for Food Safety and Hygiene, University Zurich, Zurich, Switzerland, O serotyped and MLST typed by Center for Food Safety and Applied Nutrition FDA, Laurel, Maryland, USA

[c, d]: O (Sero)type and MLST Sequence type designations retrieved from Ogrodzki and Forsythe [27].

n. a.: not applicable

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manufacturer's instructions. For purification purposes (PCR, restriction digestion, agarose gel purification) the Qiagen MinElute PCR Cleanup kit or MinElute Gel Purification kit was employed. Enzymes and respective buffers were obtained from Roche Molecular Diagnostics and used according to the manufacturer`s instructions.

Construction of *C. turicensis* LMG 23827^{T}Δ*zpx* and complementation

Bacterial strains, plasmids and primers used for the construction of the mutant are listed in Table 2. An isogenic mutant of the *C. turicensis* LMG 23827^T devoid of the *zpx* (CTU_31020) gene was constructed following the protocol described by Philippe et al. [29]. Primers were designed based on the whole genome sequence of *Cronobacter turicensis* LMG 23827^T (RefSeq accession numbers NC_013282 to NC_013285, GenBank accession numbers FN543093 to FN543096). Briefly, two flanking fragments (upstream, downstream) of the *zpx* gene were amplified by PCR using oligonucleotide primers zpxF1f (containing a *XbaI* recognition site),

Table 2. Material used for *zpx* mutant construction and complementation.

Strains/plasmids/primers	Genotype/characteristic(s)/sequences	Source or reference
Mutant construction strains		
C. turicensis LMG 23827^T_Nal^R	Acceptor for transconjugation, Nal^R	[30]
E. coli SM10 λpir	Host for pDS132::Δ*rpfF*, pDS132::Δ*rpfR* construct generation; *thi*, *thr*, *leu*, *tonA lacY supE recA*::RP4-2-Tc::Mu, Km, *λpir*	[31]
E. coli DH5α λpir / pDS132	Host for cloning vector pDS132; *sup* E44, Δ*lacU*169 (Φ80*lacZ*ΔM15), *recA1*, *endA1*, *hsdR17*, *thi-1*, *gyrA96*, *relA1*, *λpir*, Cam^R	[32]
E. coli SM10 λpir / pDS132::Δ*zpx*	Donor for transconjugation, harbouring construct pDS132::Δ*zpx*, Cam^R	This study
Plasmids		
pDS132	Low copy cloning vector R6K *ori*, *mobRP4*, *cat*, *sacB*, Cam^R	[29]
pDS132::Δ*zpx*	Δ*zpx* cloned into pDS132, Cam^R	This study
Primers		
zpxF1f	5`– AGC TCT AGA AGC GGT CGG AAG AGC CTT TGG–3`	This study
zpxF1r	5`– TAT CTC GAG GCC ATG ATC GAT AAT GCG GCG–3`	This study
zpxF2fmod	5`– GGG CTC GAG GCT CAC TCT CGC AGA ATG CGG–3`	This study
zpxF2r	5`– TGA TCT AGA GGT CTG GTG CTG GTT CAT ACC–3`	This study
zpxConf	5`– CTA TAC TGC AAG TGT TGG–3`	This study
zpxConr	5`– CGT CAT CCG TCA GAT CTG–3`	This study
Complementation		
C. turicensis LMG 23827^T	Template for amplification of *zpx* CDS	[33]
C. turicensis LMG 23827^{T}Δ*zpx*	Zpx CDS mutant, cloning host for pQE-30, pQE-30::*zpx*	This study
C. turicensis LMG 23827^{T}Δ*zpx* / pQE-30	Mutant transformant harbouring expression cloning vector pQE-30, Amp^R	This study
C. turicensis LMG 23827^{T}Δ*zpx* / pQE-30::*zpx*	Mutant transformant harbouring construct pQE-30::*rpfF*, Amp^R	This study
Plasmid		
pQE-30	Cloning/expression vector, Amp^R	Qiagen
Primers		
rpfFComplf	5`– AAA GCA TGC AAC CAG TCA CGT TAT CCA ACC– 3`	This study
rpfFComplr	5`– AAA CCC GGG CGT CGG CGT CAT CCG TCA GAT CTG–3`	This study
Insert control		
pQE-30mcsf	5`– CGG ATA ACA ATT TCA CAC AG– 3`	Qiagen
pQE-30r	5`– GTT CTG AGG TCA TTA CTG G– 3`	Qiagen

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zpxF1r (containing a *XhoI* restriction site), zpxF2fmod (containing a *XhoI* recognition site), zpxF2r (containing a *XbaI* recognition site). The amplification mixes contained 0.4 μM of primers, 1 x AccuPrime (Invitrogen) buffer 2 (60 mM Tris-SO_4 (pH 8.9), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, thermostable AccuPrimeTM protein, 1% glycerol), 4% dimethylsulfoxid (DMSO), 2 U AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen) and 50 ng of template DNA. Following PCR conditions were used for the amplification: 95°C for 120 s followed by 34 cycles of 95°C for 30 s, 68°C for 270 s and a final elongation step at 68°C for 300 s. The resulting fragments were digested with *XbaI* and *XhoI* and ligated into the suicide vector pDS132 digested with *XbaI*. The construct pDS132::Δ*zpx* was transformed into *E. coli* SM10 λpir via electroporation. The resulting strain *E. coli* SM10 λpir/ pDS132::Δ*zpx* served as donor strain for conjugative transfer of the plasmid into *C. turicensis* LMG 23827^T_Nal^R. Transconjugants were selected on LB agar plates supplemented with both nalidixic acid 256 mg L^{-1} and chloramphenicol 30 mg L^{-1}. The genetic structure of the mutant was confirmed by the presence of two amplification products—one representing the chromosomal wild type *zpx* allele and a second product representing the truncated (Δ*zpx*) allele originating from the (integrated) pDS132::Δ*zpx*—after PCR using primer pair zpxContf, zpxContr employing the above mentioned AccuPrime amplification mixture (without DMSO) and following amplification conditions: 95°C for 120 s followed by 32 cycles of 95°C for 30 s, 52°C for 210 s and a final elongation step at 68°C for 300 s. The resulting amplification products were 1101 bp (wt *zpx* allel) and 240 bp (Δ*zpx* allel).

Outcrossing was performed by plating serial dilutions of confirmed transconjugants onto LB agar plates supplemented with 5% sucrose and no NaCl. Successful allelic exchange was verified in selected chloramphenicol sensitive and and sucrose resistant strains by the presence of the mutant allele after PCR using the above mentioned procedure.

For complementation the *zpx* gene was amplified using primers zpxComplf and zpxComplr (containing an *SphI* and *XmaI* site respectively) and using the above mentioned Accuprime mixture (without DMSO) and following conditions: 95°C for 120 s followed by 32 cycles of 95°C for 30 s, 64°C for 30 s, 68°C for 120 s and a final elongation step at 68°C for 300 s. The resulting fragments were cloned into pQE-30 expression vector and the resulting plasmid pQE-30::*zpx* transformed into *E. coli* Xl1blue or *C. turicensis* LMG 23827^{T}Δ*zpx*.

Construction of *C. sakazakii* ATCC BAA-894Δ*cpa* and complementation

The construction of the *cpa* isogenic mutant, was completed according to the method published by Franco et al. [34]. For complementation experiments, the *cpa* was cloned into pQE-30 expression vector (Qiagen) by PCR amplification using the primers *XmaI*, (5`- AAA CCC GGG AAT AAG AAA CTT ATT GTC GTG GCG- 3`) and *SalI*, (5`- AAA GTC GAC AAC CCG CCG GCA GCG GG- 3`). The recombinant plasmid was transformed into *E. coli* Xl1blue or *C. sakazakii* ATCC BAA-894Δ*cpa*.

Zebrafish infection studies

Zebrafish (*Danio rerio*) strains used in this study were albino lines. Husbandry, breeding and microinjection of approx. 50 CFU of bacteria into the yolk sac of 2 dpf embryos was performed following the procedure described in the study by Fehr et al. [24]. A total of thirty embryos (10 x 3) were injected per individual experiment (i.e. per strain).

A set of uninjected embryos, incubated in E3 maintenance medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) was included in order to determine the quality of the embryos; embryos injected with DPBS served as controls. Prior to injection, embryos were manually dechorionated and anesthetized with 200 mg L^{-1} buffered tricaine (Sigma-Aldrich).

After injection, the infected embryos were allowed to recover in a petri dish with fresh E3 medium for 15 min. Injected embryos were transferred into 24-well plates (1 embryo per well) in 1 ml E3 medium per well, incubated at 28°C and inspected for survival under a stereomicroscope. At regular time points after infection, the number of dead larvae was determined visually based on the absence of a heartbeat. Experiments were carried out until 96 hours post infection (hpi). Surviving embryos were euthanized with an overdose of 4 g L^{-1} buffered tricaine at the end of the experiments. Generally, with the assessment of discomfort and pain by behavioral observations, animals were euthanized by overdose of tricaine by prolonged immersion and were left in the solution for at least 10 minutes following cessation of opercular movement. Since pain perception has not been developed at these earlier stages (4 dpf– 7 dpf), this is not considered a painful procedure.

The maximum age reached by the embryos during experimentation was 144 hpf. Embryos had not reached free feeding stage then. In addition, research was conducted with approval (NO 216/2012) from the Veterinary Office, Public Health Department, Canton of Zurich (Switzerland) allowing experiments with embryos and larvae older than 120 dpf. The applied methods were carried out following the approved guidelines.

Survival assay

Thirty 2 dpf embryos were microinjected as mentioned above and maintained individually in 24-well plates in E3 medium at 28°C. Growth behaviour of strains *C. sakazakii* ATCC BAA-894 and *C. sakazakii* ATCC BAA-894Δ*cpa* was monitored until 72 hpi.

Bacterial enumeration by plate counting

The larvae were transferred to 1.5 ml centrifuge tubes and disintegrated by repeated pipetting and vortexing for 3 min in 1 mL of DPBS supplemented with 1% Triton X- 100 (Sigma-Aldrich). Subsequently, serial dilutions of this mixture were plated onto LB plates supplemented with 256 mg L^{-1} nalidixic acid. The plates were incubated up to 48 h at 37°C.

Statistical analysis

Kaplan Meier survival analysis, log rank (Mantel-Cox) test and graphs were performed using GraphPad Prism 6 (GraphPad Software, San Diego, USA). Experiments were executed in triplicates (i.e. 10 embryos x 3 per bacterial strain).

Results

Pathogenicity varies within the *Cronobacter* genus and among strains

Infection experiments using 10 strains—the type strains of the 7 species and 2 subspecies within the *Cronobacter* genus plus one additional *C. sakazakii* strain ATCC BAA-894—revealed a substantial degree of variation as determined from the mortality rate in the embryos. Hundred per cent mortality was observed within 36 hours post inoculation (hpi) in infection experiments using *C. sakazakii* ATCC 29544^T and *C. turicensis* LMG 23827^T (Fig 1A and 1B). This rate was also reached during experiments using *C. universalis* NCT9529^T and *C. dublinensis* ssp. *dublinensis* LMG 23823^T although although death of the embryos occurred at a later time point (72 hpi). For *C. sakazakii* ATCC BAA-894 the highest mortality rate (82%) was reached at 96 hpi. Also eighty per cent mortality was observed for *C. malonaticus* LMG23826^T, *C. dublinensis* ssp. *lactaridi* LMG 23825 and *C. condimenti* LMG 26249^T at 96 hpi. For *C. dublinensis* ssp. *lausannensis* LMG 23824 infection experiments, a 40% mortality rate was observed at 96 hpi and *C. muytjensii* ATCC 51329^T was completely avirulent (Fig 1A and 1B).

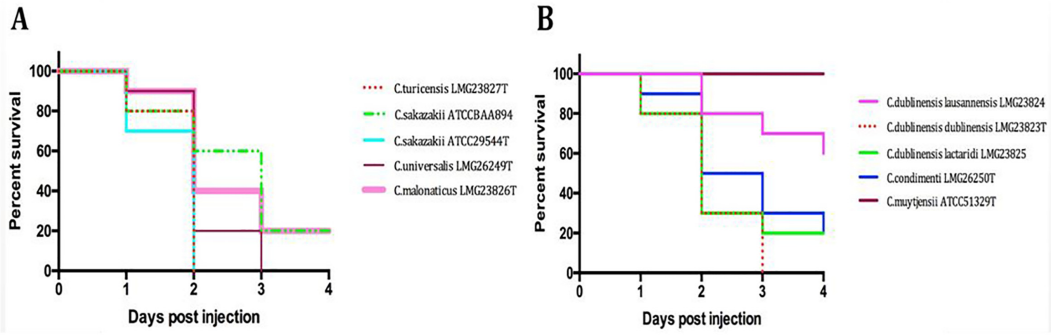

Fig 1. A, B. Zebrafish embryo infection experiments performed with *Cronobacter* wt strains. n = 30 embryos per strain, p = < 0.0001.

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The role of pESA3 and pCTU1 as "virulence plasmids" is confirmed *in vivo*

In one of the first comparative genomics studies performed on whole genomes available for the two *Cronobacter* species *C. sakazakii* ATCC BAA-894 and *C. turicensis* z3032 (= LMG 23827^T) by Franco et al. [25] it was reported that multiple plasmids were present in the strains including two homologous plasmids identified as pESA3 in *C. sakazakii* BAA-894 and pCTU1 in *C. turicensis* LMG 23827^T. *In silico* analysis revealed that both plasmids encode similar groups of genes or gene clusters comprising the "backbone"of the plasmid, the same RepFIB-like origin of replication gene (*repA*), two iron acquisition systems, an aerobactin-like siderophore (named cronobactin), and an ABC ferric-iron transporter gene cluster, as well as several species-specific virulence gene determinants. It has been proposed that these plasmids may represent "virulence plasmids"conferring virulence to *Cronobacter* isolates.

In order to test the hypothesis that these RepFIB-like plasmids harbored by *Cronobacter* spp. are involved in pathogenesis, zebrafish embryo infection studies were performed using the plasmid cured strains of *C. turicensis* LMG 23827^{T}ΔpCTU1 and *C. sakazakii* ATCC BAA-894ΔpESA3. As can be retrieved from Fig 2, the mortality of both plasmid-cured strains was considerably reduced compared to the results obtained with the wild type strains. In addition, when using *C. sakazakii* strain E899, which is naturally devoid of the pESA3 homologue in infection experiments, a maximum mortality rate of 20% was recorded at 96 hpi.

The two putative virulence factors Cpa and Zpx play a role in *in vivo* pathogenesis

In two studies by Franco et al. [25, 34], *C. sakazakii* plasmid pESA3 was shown to encode the putative virulence factor *Cronobacter* plasminogen activator (*cpa*) gene locus. It was demonstrated that the outer membrane protease Cpa provides serum resistance to *C. sakazakii* ATCC BAA-894 by proteolytically cleaving complement components, as well as by activating plasminogen and inactivating the plasmin inhibitor α2-AP. To test its influence in an *in vivo* model a knock out mutant (ATCC BAA-894*Δcpa*) was constructed and tested together with the wild type strain and ATCC BAA-894*Δcpa* complemented with the *cpa* gene in trans in the zebrafish embryo model. In addition, *E. coli* Xl1blue carrying the Cpa determinant in trans was included in the experiments. As shown in Fig 3, *C. sakazakii* ATCC BAA-894Δ*cpa* displayed only 10% mortality compared to 80% mortality observed in experiments with the wild type parental strain. Virulence was partially restored to 40% with the complemented mutant. In

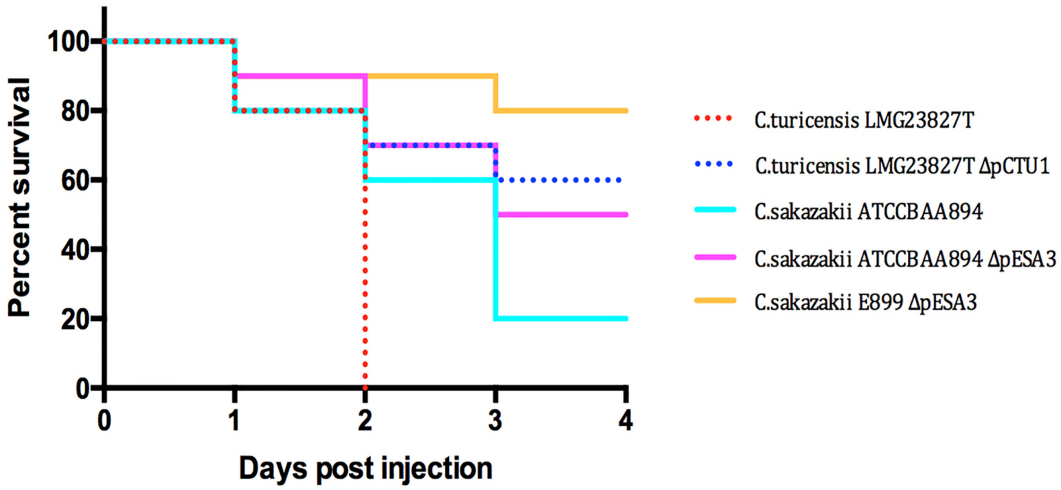

Fig 2. Infection experiments using *C. turicensis* LMG 23827^T, *C. sakazakii* ATCC BAA-894 wt, the (pESA3, pCTU1) plasmid-cured strains, and *C. sakazakii* E899 strain naturally devoid of the pESA3 plasmid. n = 30 per strain, p = 0.0007.

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addition, infection experiments using *E. coli* Xl1blue carrying the Cpa determinant displayed a moderately elevated mortality rate (20%) when compared to *E. coli* wild type strain.

In order to obtain more quatitative kinetic information on the progression of the infection, growth experiments using the BAA-894 wt and *cpa* mutant (Fig 4) were carried out. Growth of the strains in the zebrafish embryo model was comparable until 24 hpi when growth slowed in the mutant and then at 48 hpi a sharp drop was observed in the bacterial counts of the mutant suggesting that the embryos were able to clear the infection. Growth curves in LB grown cultures suggested no growth defect in the mutant (S1 Fig).

Another putative virulence factor, the zinc containing metalloprotease *zpx*, was described by Kothary et al. [35]. In contrast to the above mentioned Cpa determinant, the *zpx* locus is

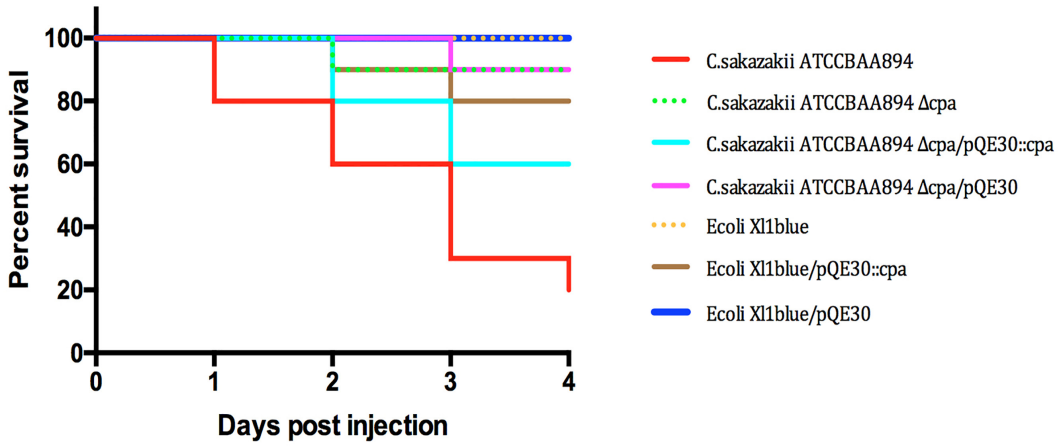

Fig 3. Infection experiments using the *C. sakazakii* ATCC BAA-894 wt, the *cpa* mutant and control strains. n = 30 embryos per strain, p < 0.0001.

doi:10.1371/journal.pone.0158428.g003

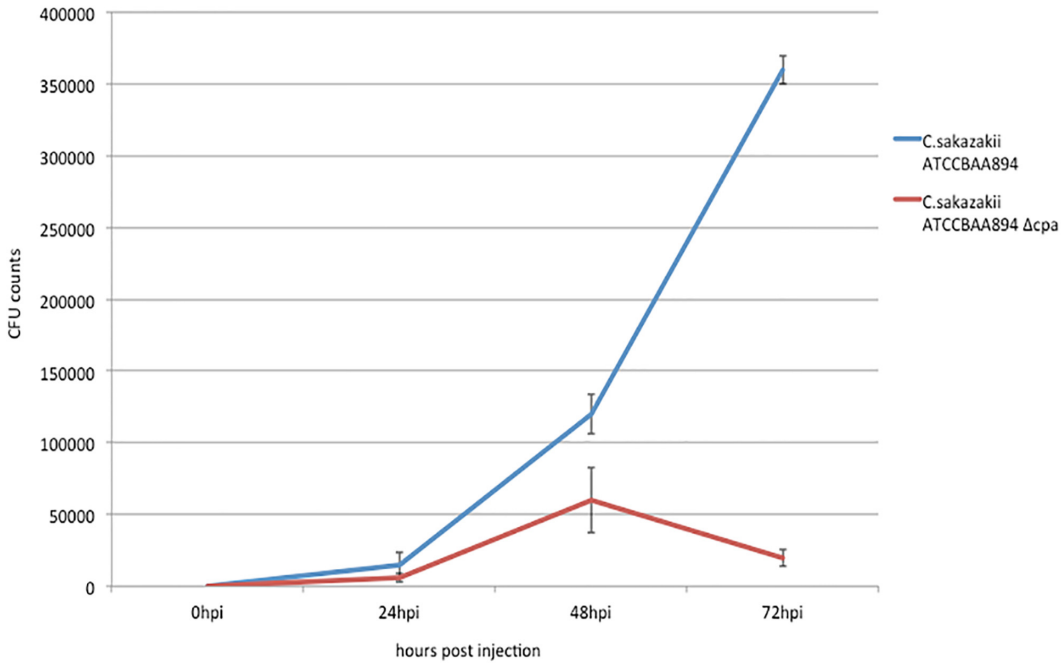

Fig 4. Quantitative infection experiments using *C. sakazakii* ATCC BAA-894 wt and the *cpa* mutant.

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chromosomally encoded and is supposed to be present in all *Cronobacter* species [31, 4]. Although activity of this protease against Chinese hamster ovary cells (CHO) in tissue culture has been reported, its contribution to virulence has never been tested before. A *zpx* mutant strain was constructed in *C. turicensis* LMG 23827^T and tested in the zebrafish embryo infection model together with its wild type, the complemented mutant and an *E. coli* Xl1blue strain carrying the Zpx determinant. The results of these experiments are depicted in Fig 5. The mortality rate in the

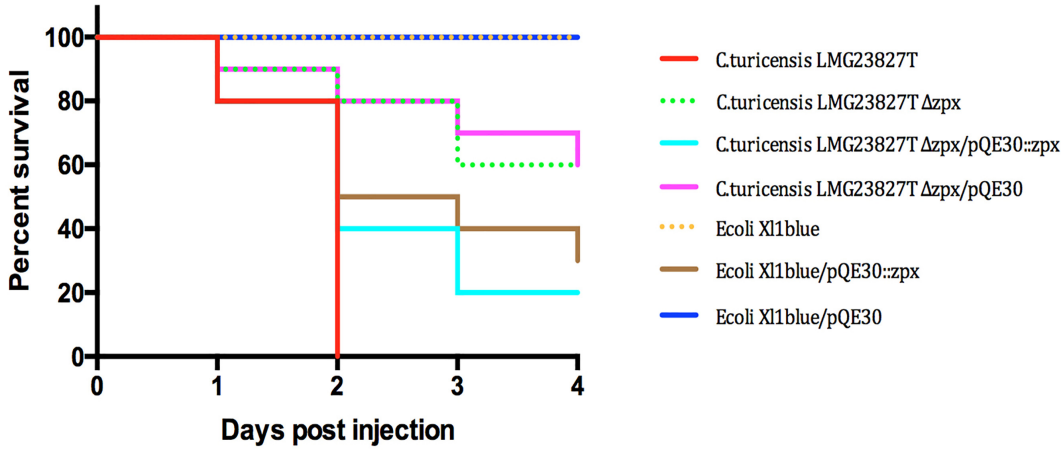

Fig 5. Infection experiments using the *C. sakazakii* ATCC BAA-894 wt, the *zpx* mutant and control strains. n = 30 embryos per strain, $p < 0.0001$.

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C. turicensis LMG 23827^T Δ*zpx* mutant was diminished by 60% and in experiments using the complemented mutant virulence was restored to a large extent (80%). As expected, the mortality rate using the *E. coli* Xl1blue harbouring the *zpx* locus was also elevated to 70%, thus underpinning the role of this important viulence factor in *Cronobacter* spp. disease.

Discussion

The results presented in this study may to some extent be correlated to the "clade theory"proposed by Grim et al. [36]. According to this hypothesis, *Cronobacter* have diverged into two clades, one consisting of *C. dublinensis* and *C. muytjensii* (Cdub-Cmuy) and the other comprised of *C. sakazakii*, *C. malonaticus*, *C. universalis*, and *C. turicensis*, (Csak-Cmal-Cuni-Ctur) from the most recent common ancestral species. It was shown, that the Cdub-Cmuy clade genomes contained several accessory genomic regions important for survival in a plant-associated environmental niche, while the Csak-Cmal-Cuni-Ctur clade genomes harbored numerous virulence-related genetic traits.

However, mortality rates such as seen with C. *dublinensis* ssp. *dublinensis* LMG 23825^T, which is supposed to be a member of the "environmental/plant associated (Cdub-Cmuy) clade"but exhibiting an equally high mortality rate as the putative "virulence associated (Csak-Cmal-Cuni-Ctur) clade"members suggests, that additional virulence factors may exist, which are more crucial for the pathogenesis/mortality (at least in this model) than others. Whole genome comparisons are currently needed in order to elucidate other potential factors that may contribute to the highly virulent phenotype observed for this strain in our model.

One striking result was the finding that infection studies performed with the *C. muytjensii* ATCC 51329^T type strain resulted in zero mortality. It has been previously reported that this strain does not harbor a RepFIB-like "virulence plasmid"[25]. The results from our experiments suggest that the presence of the these plasmid strongly contributes to virulence. Our experiments using the plasmid-cured strains compared to parental wt strains underpinned the role of these plasmids in virulence, as mortality rates were reduced by approx. half in the plasmid-cured strains *C. sakazakii* BAA-894 and *C. turicensis* LMG 23827^T. However, these strains did not become completely avirulent, again suggesting the presence of additional factors which contribute to pathogencity.

In silico analysis of pESA3 and pCTU1 revealed a high degree of similarity concerning the presence of putative virulence genes and gene clusters. However, it has also been shown, that particular virulence factors are exclusively found along species-specific evolutionary lines [25]. One of those factors—the *Cronobacter* plasminogen activator Cpa—is encoded by pESA3 and was demonstrated to be present in *C. sakazakii* strains [34]. This factor is supposed to play an important role in survival of the bacterium in the blood. Our results from the infection experiments using the isogenic *cpa* mutant confirmed an important role of this determinant in *in vivo* pathogenesis. From previous studies we know, that *Cronobacter* is not capable to replicate in blood [37] thus, for infection studies in zebrafish, bacteria have to be injected into the yolk saculum where they start to proliferate before traversing into the blood followed by dissemination into embryonic tissues- causing fatal bacteremia [20]. When we perfomed quantitative growth experiments in embryos using wt and *cpa* mutant we observed equal growth behaviour for both strains until 48 hpi. However, at later time points the bacterial counts decreased significantly in infections with the *cpa* mutant. We hypothesize that at time points $>$ 48 h post infection the bacterium had already transversed into the blood where a mutant deficient of the *cpa* determinant was efficiently eliminated by the host immune system.

In conclusion, we show, that the zebrafish embryo infection model is an extremely useful tool to study the contributions of putative virulence factors whether they have been derived by

in silico analysis or from previous *in situ* experiments. The potential of this model for use in large scale infection studies holds much promise in improving the understanding of the virulence potential of these pathogens.

Supporting Information

S1 Fig. Growth curves of the *C. sakazakii* ATCC BAA-894 wt and the *cpa* mutant gown in LB. Bacterial growth was monitored over 24 h at 37°C at 600 nm in 200 μl volumes of medium in 96 well plates using the Bio-Tek microplate reader (Synergy HT; Bio-Tek, Germany).
(TIF)

Author Contributions

Conceived and designed the experiments: AE BDT SCFN RS AL. Performed the experiments: AE JG GRG IRP. Analyzed the data: AE BDT JG GRG IRP AL. Contributed reagents/materials/analysis tools: BDT SCFN RS. Wrote the paper: AE BDT RS AL.

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Chapter 6

Listeria monocytogenes Csps Promote Virulence and Flagella-based motility Through Expression Regulation of Key Virulence and Flagella genes

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Contribution:

Planning of experiments, Cell culture, Invasion and Gentamicin assays, aggregation and motility assays, RTPCR, western blot, statistical analysis, cell staining, zebrafish experiments, confocal microscopy, image analysis, preparation of figures and graphs and writing the manuscript.

Abstract

Cold shock proteins (Csps) are small multifunctional nucleic acid binding proteins used by bacteria to regulate various gene expression events including transcription, mRNA stability and translation. We show here that Csp functions are required for the full expression of virulence and motility phenotypes in *L. monocytogenes.* Based on evaluation of *L. monocytogenes* EGDe mutants harboring triple (*ΔcspABD*) and double (*ΔcspBD, ΔcspAD* and *ΔcspAB*) deletions of *csp* genes we show that Csps are functionally necessary for growth and persistence inside human macrophages, virulence as evaluated in a zebrafish infection model, swarming motility and surface expression of flagellation in this bacterium. Furthermore, we also show that Csp contribution to these *L. monocytogenes* phenotypes is associated with Csp-dependent regulation of virulence and motility associated gene expression.

Introduction

Current serious public health and food safety problems exist due to the Gram-positive intracellular opportunistic foodborne bacterial pathogen *Listeria monocytogenes* (1-2). Listeriosis, a disease associated with severe illnesses, high mortality, abortions and stillbirths is caused by *L. monocytogenes* in susceptible immunocompromised human populations (3-4). Owing to highly adaptable physiology as well as widespread natural occurrence this bacterium also accounts for significant food safety challenges and substantial economic losses to the food industry (5-7). As such virulence and stress resilience are important *L. monocytogenes* attributes. These features depend on various molecular and physiological response mechanisms, which allow this bacterium to resist different food preservation and host defense response measures as well as facilitate invasion, survival and induction of pathogenicity in different types of mammalian host cells (5, 8-10).

Some of the key virulence factors involved in host cell infection have been illuminated in this pathogen (8-9). Entry into non-phagocytic host cells is induced using internalin proteins InlA and InlB, whereas in phagocytic host cells such as macrophages *L. monocytogenes* enters through simple phagocytosis (11-12). Internalized *Listeria* cells employ proteins such as Listerolysin O (LLO), phospholipases (PlcA and PlcB) and the metalloprotease Mpl to

escape from internalization vacuoles and phagosomes into the host cell cytosol for replication (13-16). While intracellular motility and cell to cell dissemination of *L. monocytogenes* is mediated through the surface protein ActA and InlC (17). All these different key virulence factors involved in host cell infection are transcriptionally regulated through the master transcriptional regulator protein PrfA, which is activated in *Listeria* cells upon host infection (18). In addition, *L. monocytogenes* also employs flagella and flagella-based motility to facilitate some of its virulence and stress resistance associated responses. Besides facilitating adhesion and invasion host cell, flagella and flagella-based motility is also important for cold growth as well as being involved in surface attachment and biofilm formation (19-22).

The proteins of the cold shock-domain protein family (Csps) produced in various bacteria are small highly conserved nucleic acid binding proteins that are implicated in regulation of various gene expression events (23-24). Consequently, Csps are functionally important in execution of various aspects on bacterial physiology including stress adaptation and virulence responses (23-24). Originally discovered in connection with cold adaptation, it subsequently emerged that Csps have a wide range of roles in bacteria during normal growth as well as in various stress adaptation responses and virulence associated phenotypes (25-27). Exact mechanisms of gene expression regulation through Csps are not yet fully understood. But they are presumed to involve DNA and RNA binding events that allow these proteins to regulate transcription, mRNA stability, translation, DNA replication and chromosomal condensation processes (25,28-29).

L. monocytogenes produces three highly conserved Csps, CspA, CspB and CspD. Some Csps were originally observed to be induced in response to cold, HHP and NaCl salt stresses suggesting that these proteins were functionally required during *L. monocytogenes* adaptation against such types of food related stress conditions (31-32). Subsequently Csps were confirmed to be functionally required in adaptation of this bacterium against cold and salt (NaCl) stress and oxidative stress protection (30-33). In addition, the Csps are also functionally important for the full exhibition of some virulence phenotypes in this bacterium. *L. monocytogenes* mutants devoid of all three Csps or lacking CspB and CspD are diminished in human intestine epithelial cell invasion, murine macrophage growth and LLO

production capacities (33-34). In this study we report on further characterization of Csp roles in *L. monocytogenes* virulence and flagella-based motility associated phenotypes. Based on evaluation of *L. monocytogenes* EGDe mutants harboring triple (*ΔcspABD*) and double (*ΔcspBD, ΔcspAD* and *ΔcspAB*) deletions of *csp* genes we show that Csps are functionally necessary for growth and persistence inside human macrophages, virulence as assessed in a zebrafish multicellular infection model, swarming motility and surface expression of flagellation in this bacterium. Furthermore, we also show that Csp contribution to these *L. monocytogenes* phenotypes is associated with Csp-dependent regulation of virulence and motility associated gene expression.

Materials and Methods

Strains and growth conditions

Wild-type (WT) and corresponding *csp* gene family deletion mutant strains of *L. monocytogenes* EGDe used in this study are described in Table 1. All the *csp* gene deletion mutants were constructed in-frame as previously described (30). Green fluorescent protein (GFP) expressing derivatives were generated using site specific PSA-integrase mediated single copy integration of the pPL3-eGFP plasmid in EGDe WT and *csp* deletion mutant strains (35) into the tRNA-Arg locus (Lauer et al., 2002). For cultivation, strains were grown in 10 ml Brain-Heart Infusion (BHI; Oxoid, Hampshire, UK) broth cultures, overnight (16 h) at 37°C with gentle (150 rpm) shaking. Where appropriate, culture medium or agar was supplemented with erythromycin at 5µg ml^{-1} (pPL3-eGFP integrated strains). Bacteria for microinjection experiments were harvested by centrifugation at 5000x g for 10 min and washed once in 10 ml DPBS. After a second centrifugation step the cells were resuspended in DPBS and appropriate dilutions were prepared in DPBS.

THP1 cell culture

THP-1 cells (ATCC TIB-202) were seeded onto T75 tissue culture flasks (TPP- Techno plastic products, Switzerland) and grown to confluence in RPMI 1640 medium (Sigma Aldrich, Germany) containing 0.3g l^{-1} L-glutamine, 2g l^{-1} sodium bicarbonate supplemented with 10 mM HEPES (Sigma Aldrich, Buchs, Switzerland), 1 mM sodium pyruvate (Sigma Aldrich, Buchs, Switzerland), 4.5 g l^{-1} glucose and 10 % fetal bovine heat-treated (56 °C, 30 min)

serum (Sigma Aldrich, Buchs, Switzerland Germany) and incubated at 37 °C with 5 % CO_2. THP-1 cells were seeded on to 24-well plates at 1 x 10^5 cells per well in RPMI 1640 medium supplemented with 0.1 µg ml-1 of phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, Buchs, Switzerland) at 37^{o}C with 5 % CO_2 for at least 24 h prior to infection. The PMA treatment caused the THP-1 cells to become adherent and activated and has been shown to induce stable differentiation of THP-1 monocytes into macrophages without undesirable gene upregulation.

Invasion/gentamicin protection assays

Prior to infection medium containing PMA was removed and cells were gently washed with fresh RPMI 1640 medium to remove residual PMA and substituted with 500 µl per well of fresh RPMI 1640 medium without PMA. Sixteen-hour stationary phase *Listeria* (≈10^9CFU/ml) cultivated in BHI broth at 37 °C (150 rpm shaking) were diluted in RPMI (≈10^8CFU/ml) and used for infection assays. THP-1 macrophages were infected with an MOI of 1:100 (≈10^7 CFU per well) and incubated for 45 min at 37 °C with 5 % CO_2. Following incubation media was replaced with 500 µl of fresh RPMI 1640 medium containing 100µg ml^{-1} gentamicin. Plates were incubated for 45 min under the above-mentioned conditions to kill extracellular bacteria. Infected macrophages were subsequently washed twice gently with 1 ml of DPBS (Dulbecco's Phosphate-Buffered Saline). 0.5 % Triton X-100 (Sigma) in DPBS was added to lyse the infected macrophages. Appropriate 10-fold serial dilutions of the macrophage lysates were plated on plate count agar plates (PC agar, Sigma) and incubated 24h at 37°C to determine the number of viable bacteria via plate count. Number of viable bacteria was presented as the percentage of the inoculum that is intracellular. This time point is represented as t0.

For extended (intracellular survival/replication) assays the 100µg ml^{-1} gentamicin containing media was removed. Macrophages were washed with fresh medium and maintained in medium containing 10 µg ml^{-1} gentamicin during incubation for 1 (t1), 4 (t4), 6 (t6), 24 (t24) and 48 (t48) hours before washing, lysis and total viable bacteria counting on PC agar as mentioned above.

Results are presented as percent survival of the initial intracellular population recovered at time zero for each indicated time point. For all assays three experiments were performed on three independent occasions.

Confocal laser scanning microscopy

GFP expressing EGDe WT and *csp* mutant strains (Table 1) were used in macrophage infection assays with subsequent microscopic analysis. In each well of the 24 well plate, a sterile glass coverslip (13 mm in diameter, Menzel-Gläser) was placed and THP-1 cells were seeded on the coverslips. Cell culture, PMA activation and infection assays were carried out as mentioned above. At each indicated time point, THP-1 macrophages adhered to coverslips were gently washed twice with DPBS or RPMI medium, fixed with 4 % para formaldehyde (PFA, Sigma) at 4 °C for 15 min. Following fixation PFA was removed and macrophages were gently washed with DPBS to remove residual PFA and stained with 3. 5 µl of 3 µg ml^{-1} Hoechst Dye (Life Technologies, Zug, Switzerland) and 5µl of 5 mg ml^{-1} Concanavalin A Alexa Fluor® 594 Conjugate (Life Technologies) and incubated at room temperature for 1 h. After incubation macrophages were washed 3-5 times with DPBS, mounted on glass slide using Fluoromount (Sigma) mounting medium, air dried in dark and imaged under Leica TCS SP5 Confocal microscope (63x or 40x oil-immersion objective, excitation at 405 nm for Hoechst, 488 nm for GFP and 594 nm for Concanavalin A).

Zebrafish lines and husbandry

Zebrafish (*Danio rerio*) strains used in this study were predominantly *wik* lines. Adult fish were kept at a 14/10 hours light/dark cycle at a pH of 7.5 and 27°C. Eggs were obtained from natural spawning between adult fish which were set up pairwise in individual breeding tanks. Embryos were raised in petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) supplemented with 0.3 µg/ml of methylene blue at 28°C. From 24 hpf, 0.003% 1-phenyl-2-thiourea (PTU) was added to prevent melanin synthesis. Staging of embryos was performed according to Kimmel et al. Research was conducted with approval (no. 216/2012) from the Veterinary Office, Public Health Department, Canton of Zurich (Switzerland).

Microinjection experiments

Injections were performed using borosilicate glass microcapillary injection needles (Science Products, 1210332, 1 mm O.D. x 0.78 mm I.D.) and a PV830 Pneumatic PicoPump (World Precision Instruments). Two dpf embryos were manually dechorionated and anesthetized with 200 mg/l buffered tricaine (MS-222) prior to injections. Subsequently embryos were aligned on an agar plate and injected with 100 CFU (range 90 - 136CFU) in 1-2 nl volume of a bacterial suspension in DPBS into the blood circulation via the caudal vein close to the urogenital opening or into the yolk sac. Prior to injections the volume of the injection suspension was adjusted by injecting a droplet into mineral oil and measuring its diameter over a micrometer scale bar. The number of CFU injected was determined by direct microinjection of a DPBS droplet on agar plates and confirmed by disintegrating 5 embryos individually immediately after microinjection (0 hpi) and plating the lysates on LB agar. Post injection the infected embryos were allowed to recover in a petri dish in fresh E3 medium for 15 min. To follow the infection kinetics and mortality, embryos were transferred into 24-well plates (one embryo per well) in 1 ml E3 medium per well, incubated at 28°C and observed for signs of disease and survival under a stereomicroscope twice a day. For survival assays after infection, the number of dead larvae was determined visually based on the absence of a heartbeat. At each time point, five embryos or larvae were collected and individually treated for bacterial enumeration. For subsequent microscopic analyses larvae were euthanized with an overdose of 4 g/l buffered tricaine and transferred into respective buffers and fixatives.

Bacterial enumeration by plate counting

The larvae were transferred to a 1.5-ml Eppendorf tube containing 1 ml DPBS supplemented with 1% Triton X- 100 and disintegrated by repeated pipetting and vortexing for 3 minutes. Subsequently, 100 µl of this mixture was plated onto LB selective plates (i.e. erythromycin 5 µg l^{-1} for strains harbouring pPL3::GFP) and plates were incubated up to 48 h at 37 °C.

RNA extraction and RTPCR

Reverse transcription quantitative-PCR (RT-qPCR) was used to determine the expression levels of genes in EGDe WT and *csp* mutants. Organisms grown to stationary phase in BHI

broth culture and those recovered from infected macrophages were used. *Listeria* cells in 1 ml of broth culture were harvested in RNA protect Bacteria reagent and resuspended using 0.5 ml of the RNeasyPlus Mini Kit (Qiagen) lysis buffer. THP-1 macrophages infected for 6h with *Listeria* were washed and lysed as described above. *Listeria* released into the macrophage lysates were similarly harvested in RNA protect Bacteria reagent and re-suspended in lysis buffer. *Listeria* cells in the lysis buffer were transferred into beads in MaGNA lyser tubes and mechanically disrupted using the MagNA Lyser Instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland). RNA was subsequently isolated from the lysates following the RNeasy Plus Mini Kit protocol (Qiagen, Hombrechtikon, Switzerland). Purified RNA was quantified in the Nanodrop (Thermo scientific, USA) and its quality was verified using the BioAnalyzer (Aglient technologies, USA). cDNA was synthesized from 100 ng of RNA using the Quantitect Reverse Transcription Kit (Qiagen, Hombrechtikon, Switzerland). Residual DNA contamination of RNA samples was ruled out through inclusion of no RT controls in the analysis. 2.5ng of the cDNA were used as templates for quantitative real-time PCR (qRT-PCR). Primers listed in Table 1 and the SYBR green I kit (Roche Molecular Diagnostics, Penzbrug, Germany) were used for quantitative real-time PCR in the LC480 (Roche Molecular Diagnostics, Rotkreuz, Switzerland) instrument. Quantification was performed using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics) and mRNA levels were normalized using 16S rRNA as reference gene. Transcript levels of each gene were expressed relative to the values of a calibrator mRNA sample, which was derived from stationary phase EGDe WT culture grown in BHI broth.

Western blotting

Total cell protein extracts were generated from BHI broth cultivated and macrophage grown *Listeria*. 30 ml of stationary phase cultures (16 h at 37°C and 220 rpm) of each strain were prepared as described above, standardized to OD_{590} of 1.0 and centrifuged (10,000×g) for 2 min at 4°C. Listeria pellets were resuspended in RIPA lysis buffer (1 ml) that contained a protease inhibitor cocktail (Cell Biolabs Inc, San Diego, USA) and mechanically disrupted (2 x 6500 rpm for 1 minute) in the MaGNA lyser instrument (Roche Molecular Diagnostics). Intracellular *Listeria* were released following lysis of THP-1 macrophages infected for 6h were recovered by centrifugation and mechanically disrupted. Cell debris in the lysates was

centrifuged and proteins contained in supernatants were collected into fresh tubes and their concentration was determined. Equal protein amounts from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 10%) and transferred onto PVDF membranes. The membranes were probed with primary rabbit polyclonal antibodies raised against PrfA, Mpl and LLO, as well as a primary mouse monoclonal directed against ActA. A HRP-conjugated anti-rabbit IgG secondary antibody was subsequently used to probe all except for the ActA blot, which was probed with a HRP-conjugated anti-mouse IgG secondary antibody.

Detection of PI-PLC activity and bacterial aggregation assays

To compare PI-PLC activities, colonies of EGDe WT and *csp* mutant strains were streaked on ALOA (Agar Listeria according to Ottaviani and Agosti) plates, incubated at 37°C and visually examined after 48 h for the zone of opacity. The abilities of EGDe WT and corresponding *csp* mutant strains to aggregate was compared in BHI essentially as previously described (ref). Sixteen-hour stationary phase cultures from each strain were adjusted to OD_{590} of 3.0. The samples statically incubated over 24 h at 37°C during which aliquots were collected approximately 1 cm from the top of each sample at defined time intervals to measure OD_{590}.

Swarming motility assay

EGDe WT and *csp* mutant strains were spotted on 0.25% BHI agar and incubated at 25°C for 48 h, and levels of swarming motility was observed.

Electron microscopy

Bacteria were grown overnight at 25^0 C on a 0.25% BHI agar plate. Bacterial colonies were picked up using an inoculation loop and transferred to an Eppendorf tube containing 100ul of 2.5% glutaraldehyde and incubated for atleast 30 min at room temperature to fix. Further, 1:1 suspension was prepared using 0.1M sodium phosphate buffer and bacteria-glutaraldehyde mix. This suspension was then transferred onto 150 mesh copper grids with Formvar carbon film (Electron Microscopy Sciences, USA) and incubated at room temperature for 2 min for adhesion. Following adhesion, bacterial flagella were negatively

stained with uranyl acetate for 10 seconds and with 1:1 suspension of uranyl acetate and water for 20 secs, and observed in an electron microscope.

Statistical analysis

Statistics for experiments with Zebrafish was done with GraphPad Prism 6 (GraphPad Software, United States). All graph designs were done with Microsoft Excel software. Experiments were performed at least three times, unless stated otherwise. At various time points and under various conditions numbers of CFU of groups of individual larva were tested for significant differences by one-way ANOVA with Bonferroni's posttest. ***, $p<0.001$; ns, not significant. After 45 min co-incubation of bacteria with macrophages, followed by 45min 100µg gentamicin treatment, the number of bacteria that is intracellular is presented as time point zero (t0). Gentamicin protection assay was performed at several time points up to 96 h (t96) to assess bacterial survival or replication within macrophages. Results are presented as percentage of initial inoculum that was intracellular at time point zero. Three independent assays were performed in triplicate and data obtained are means of ± standard error. The gene expression data from *Listeria monocytogenes* EGDe and *csp* mutant strains was log converted and compared using t-tests, where P-values <0.05 were considered to be statistically significant.

Table 1. Strains and plasmids used in this study.

Strains	Description	References
***L. monocytogenes* EGDe strains**		
EGDe WT	Wild type, serotype 1/2a, ATCC BAA-679	Glaser et al., 2001
EGDe Δ*cspABD*	In-frame deletions of *cspA, B and D*	Schmid et al., 2008
EGDe Δ*cspBD*	In-frame deletions of *cspB* and *D*	Schmid et al., 2008
EGDe Δ*cspAD*	In-frame deletions of *cspA* and *D*	Schmid et al., 2008
EGDe Δ*cspAB*	In-frame deletions of *cspA* and *B*	Schmid et al., 2008
EGDe WT::pPL3-GFP	pPL3-chrosomally integrated	This study
EGDe Δ*cspABD*::pPL3-GFP	pPL3-chrosomally integrated	This study
EGDe Δ*cspBD*:: pPL3-GFP	pPL3-chrosomally integrated	This study
EGDe Δ*cspAD*:: pPL3-GFP	pPL3-chrosomally integrated	This study
EGDe Δ*cspAB*:: pPL3-GFP	pPL3-chrosomally integrated	This study
Plasmids		
pPL3-GFP	pPL3-eGFP (constitutive)	Shen and Higgins, 2005

Table 2. Primers used in this study.

Gene target	Primer sequence (5`- 3`)	Protein
16S rDNA	CTT CCG CAA TGG ACG AAA GT CTC ATC GTT TAC GGC GTG	Small Ribosomal RNA subunit
mpl	TCA GGT GCG CTA AAC G GTC GCC TTC CTC TGT G	Metalloprotease
actA	GCA CCG GCT CTG ATA AG GGT AGG CTC GGC ATA TT	Actin assembly-inducing protein (ActA)
plcA	TCGGGGAAGTCCATGA GGCGCACCTAACCAAG	Phosphatidylinositol phospholipase C (PI-PLC)
plcB	TCCAGGCTACCACTGT CCAGTAGGTTCCACTGT	Phosphatidylinositol phospholipase C (PI-PLC)
hly	ACCTCGGAGACTTACG TCCTCCAGAGTGATCG	Listeriolysin O (LLO)
prfA	TGGTATCACAAAGCTCACG GACCGCAAATAGAGCC	Positive regulatory factor A (PrfA)

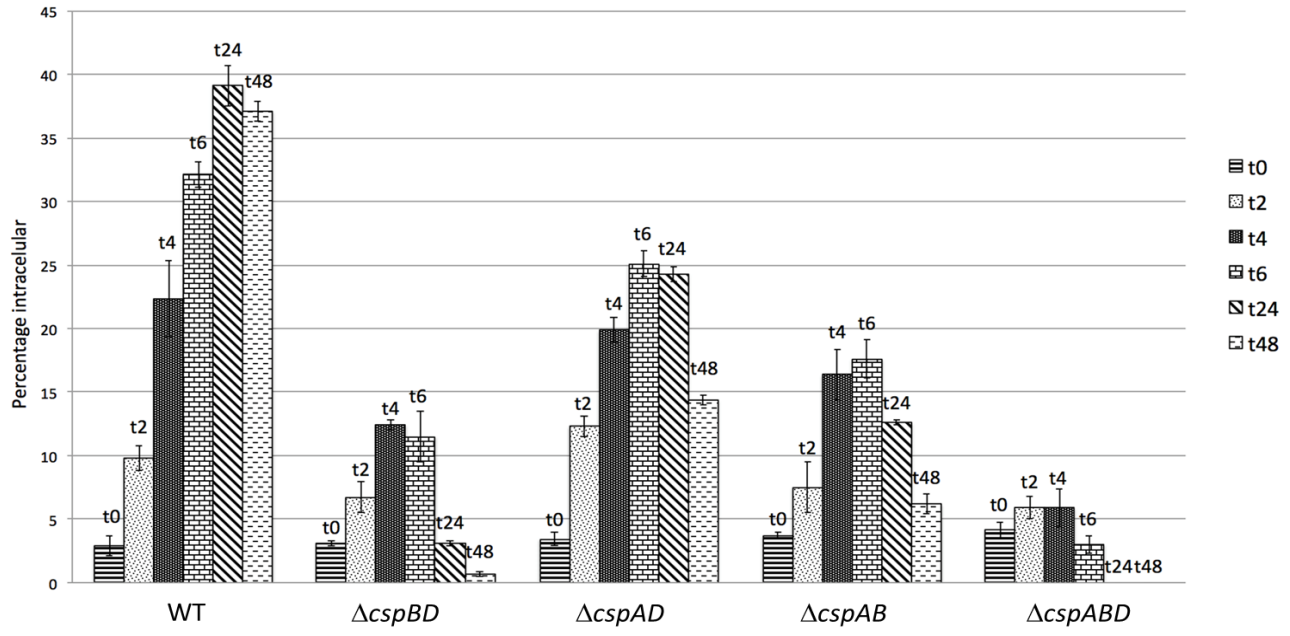
Results

Growth and persistence of *L. monocytogenes csp* mutants in human macrophages is diminished.

Compared to its parental WT strain of *L. monocytogenes* EGDe, a triple *csp* gene deletion mutant devoid of Csps, the *ΔcspABD* strain, was severely attenuated in growth and persistence inside human derived THP-1 macrophages. These defects were confirmed based on assessing viable intracellular bacteria and monitoring the intracellular fate of GFP tagged organisms during THP-1 macrophage infections. While assessing viable intracellular bacteria counts showed that the WT grew and persisted over 48 hrs, the *ΔcspABD* mutant showed limited growth getting completely cleared from THP-1 macrophages within 24 hrs of infection (Fig 1A). Fluorescent microscopy imaging of GFP-tagged intracellular organisms

during these THP-1 macrophage infections showed that despite the fact that the WT and *ΔcspABD* strains were similarly internalized as observed 1 h post macrophage infection, there were stark differences in intracellular bacteria growth and amounts observed after 6 and 24 hrs of infection (Fig. 1B). Several infected macrophages characterized by high intracellular bacteria amounts were observed with the WT strain at both time points. In contrast there were few infected macrophages characterized by low intracellular bacteria amounts observed at 6 h post-infection, and there were no macrophages containing bacteria detected at 24 h of infection with the *ΔcspABD* strain. To assess specific roles for individual Csp variants without interference from the other Csps due to functional redundancy, the *ΔcspBD*, *ΔcspAD* and *ΔcspAB* strains harboring only CspA, CspB and CspD proteins, respectively, were similarly examined. All three Csps variants produced individually conferred variable growth and persistence phenotypic levels in human macrophages that were superior to the Csp devoid *ΔcspABD* strain but below WT levels (Figs. 1A and 1B). Thus while all three Csps seem to have some functional relevance in enabling growth and persistence of *Listeria* inside human macrophages, they are not all functionally equivalent in their roles. Individual Csps displayed an apparent hierarchy with respect to their functional impacts on macrophage growth and persistence phenotypes exhibited (Figs. 1A and 1B). CspB (*ΔcspAD*) conferred the highest levels whereas CspA (*ΔcspBD*) conferred the lowest levels of macrophage growth and persistence phenotypic complementation. Thus overall our results confirm the functional relevance of three Csp variants in promoting growth and persistence of *L. monocytogenes* inside human macrophages while ranking them in order of CspB (*ΔcspAD*)>CspD (*ΔcspAB*)> CspA (*ΔcspBD*) in the roles in this process.

A



B

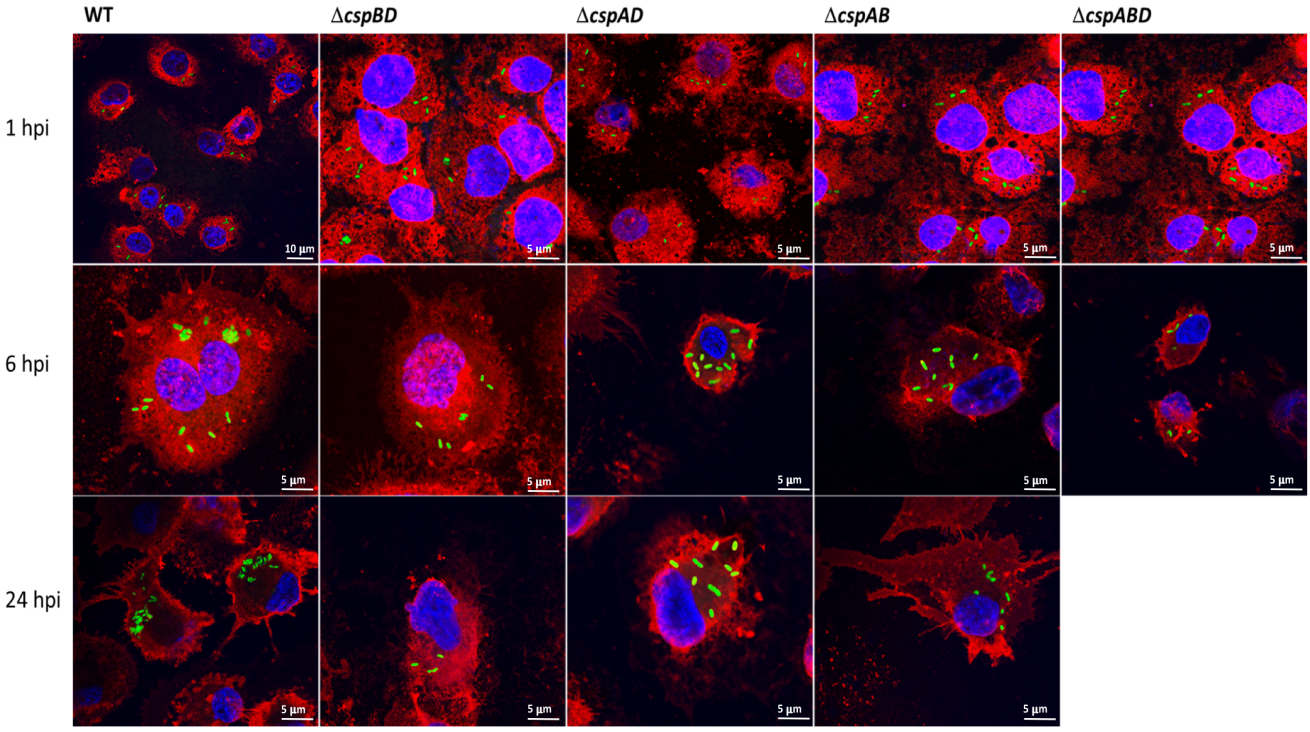


Figure 1. Impact of Csp loss on growth and persistence in human derived THP-1 macrophages. (A) Viable bacterial cell count based bar charts depicting intracellular growth and persistence of *L. monocytogenes* EGDe WT and *csp* mutants (*ΔcspBD*, *ΔcspAD*, *ΔcspAB*

and *ΔcspABD*) during 48 hours of macrophage infection. Results represent the mean (bar charts) and standard deviation (error bars) of three biological repeats. (B) Representative images (63x or 40x magnification) from monitoring the intracellular fate of GFP-labelled EGDe WT and *csp* mutants (*ΔcspABD*, *ΔcspBD*, *ΔcspAD* and *ΔcspAB*) at 1, 6 and 24 h post macrophage infection. Images presented are from one of three independent experiments.

Virulence of *L. monocytogenes csp* mutants in zebrafish is diminished.

A multicellular infection model based on zebrafish embryos was next used to assess Csp functional requirements in *L. monocytogenes* virulence. While the parental WT strain caused mortality within 48 and 24 hrs, respectively, of all (100%) zebrafish embryos infected using the blood stream and yolk sac injection routes, the *L. monocytogenes* EGDe *ΔcspABD* mutant devoid of Csps was not able to induce mortality or visible pathological changes in similarly infected zebrafish embryos (Fig. 2A and 2B). All three single Csp producing strains (Δ*cspBD*, Δ*cspAD* and *ΔcspAB*) also achieved 100% mortalities but only after 72 hrs in zebrafish embryos that were infected by the blood stream injection route (Fig. 2A and 2B). In yolk sac injected embryos on the other hand, 100% mortality occurred after 48 hrs with the CspB (Δ*cspAD*) producing strain, but the CspD (*ΔcspAB*) and CspA (Δ*cspBD*) producing strains achieved similar mortality levels after 72 h of infection (Fig. 2A and 2B). Rates of killing and pathological change developments observed in zebrafish embryos infected with the single Csp strains similarly also exhibited a hierarchichal trend of CspB>CspD>CspA, which mirrored the growth and persistence trends in human macrophages. A concurrent 48 h monitoring of bacterial loads in blood stream injected zebrafish embryos showed similar increasing trends of bacterial load in embryos infected with WT and the three single Csp strains. In contrast there was limited growth within 24 hrs and significant reduction of the initial bacterial load observed after 48 hrs (48 hpi) in embryos infected with *ΔcspABD* mutant indicating that both growth and survival of this Csp devoid mutant in zebrafish embryos was impaired (Fig. 2C). Overall our observations thus also confirmed that Csps although not functionally equivalent are necessary required for the full expression of *L. monocytogenes* virulence in this zebrafish embryo based multicellular infection model.

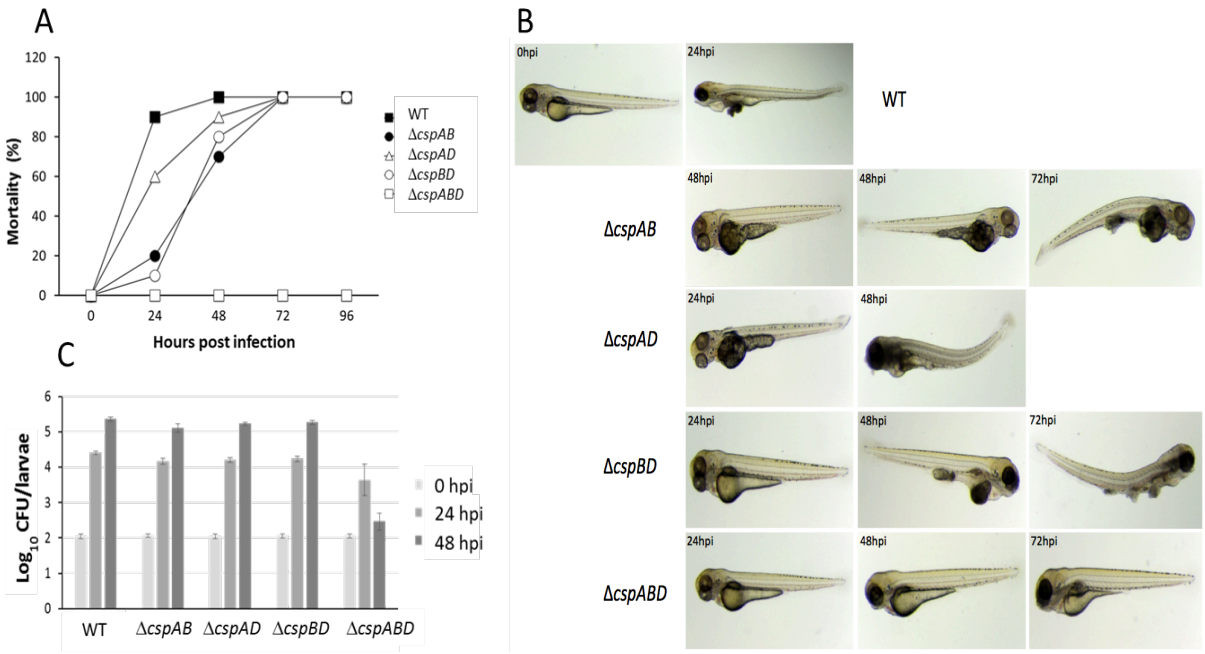


Figure 2. Csps are necessary for expression of maximal *L. monocytogenes* virulence in the zebrafish infection model. (A) Percentage mortality and (B) bacteria burden in zebrafish embryos (n=6) that were blood stream injected (100 CFU per embryo) with EGDe WT and *csp* gene deletion mutants. (C) Progressive pathological changes and mortality in zebrafish embryos (n=30) that were yolk sac injected (100 CFU per embryo) with EGDe WT and *csp* gene deletion mutants. Scale bar- 500 µm, 8x magnification.

Impaired virulence gene expression in *L. monocytogenes csp* mutants.

Virulence factors such as the PrfA, LLO, PlcA, Mpl and ActA proteins are crucial to the expression of *L. monocytogenes* virulence phenotypes. Impaired macrophage survival and replication capacity as well as zebrafish virulence displayed by the *L. monocytogenes csp* mutants thus also prompted us to examine the impact of Csp deficiency on the expression of these important virulence factors. Using Western blot analysis, we found that the PrfA, LLO, Mpl and ActA protein levels in the *csp* mutants were all lower relative to WT strain levels. This was corroborated using bacteria cultivated in BHI broth as well as organisms recovered from infected human macrophages (Figs. 3A and 3B). The Csp devoid *ΔcspABD* mutant showed the lowest PrfA, LLO and Mpl amounts compared to the WT strain (producing all three Csps) as well as the three single Csp (Δ*cspBD*, Δ*cspAD* and Δ*cspAD*)

producing strains. ActA on the other hand was undetectable in *ΔcspABD* (no Csps) and *ΔcspAB* (CspD producer) strains. In Δ*cspBD* (CspA producer) and Δ*cspAD* (CspB producer) strains it was detected but below the WT levels. PlcA amounts were compared by measuring PI-PLC activity between the strains using bacteria grown on ALOA plates. This also revealed reduced PlcA production in all the *csp* mutants compared to the WT strain (Fig. 3C). Similarly, the lowest PlcA production was detected in *ΔcspABD* that harbors no Csps, whereas the single Csp strains showed variable levels with the CspB (Δ*cspAD*) producing strain showing the highest level of activity.

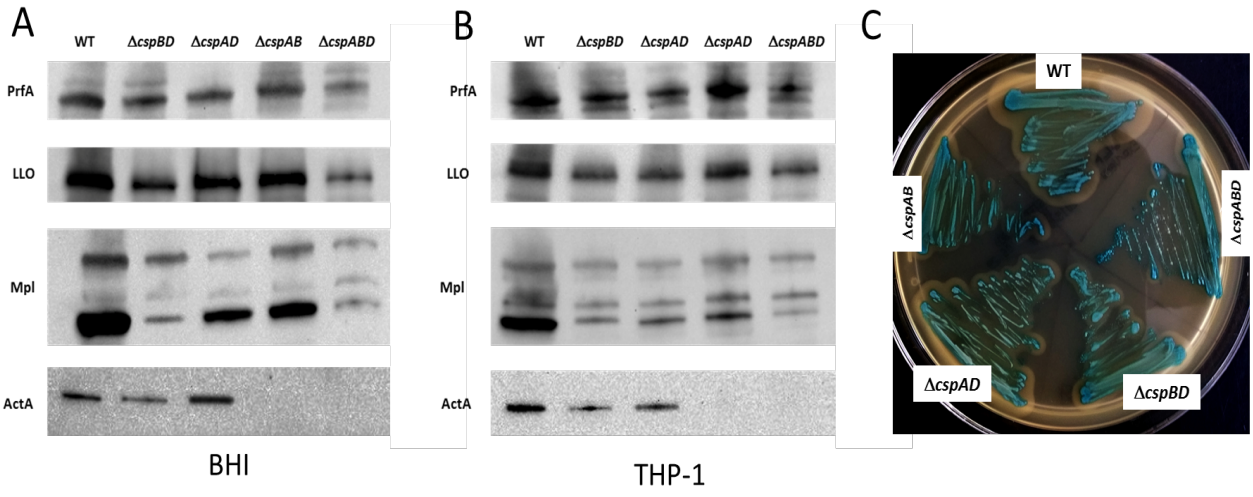


Figure 3. Csp-dependent virulence gene expression. Western blot detection of PrfA, LLO, Mpl and ActA. Equivalent total protein amounts extracted from (A) BHI (16 h culture) and (B) macrophage grown (6 h post infection) EGDe WT and *csp* mutant cell lysates were subjected to Western blot analysis. A representative of three independent experiments is presented. (C) Detection of PI-PLC (PlcA) activity in EGDe WT and *csp* mutants on ALOA plates. The turbid zone around the streaked bacteria is indicative of PI-PLC activity. Images presented are from one of three independent experiments.

Quantitative real-time RT-PCR analysis similarly revealed that the *prfA*, *hly*, *mpl* and *plcA* mRNAs encoding the PrfA, LLO, Mpl and Plc, respectively, were also significantly lower (*P<0.05*) in *csp* mutants than the WT strain (Fig. 4). In case of the three single Csp producing strains the *prfA*, *hly*, *mpl*, and *plcA* mRNA amounts were higher than those in *ΔcspABD* but

below WT levels. The level of transcripts encoding these four proteins generally followed the trend CspB>CspD>CspA. In contrast, the *actA* mRNA amounts did not reflect the ActA protein level trends. All *csp* mutants including the *ΔcspABD* and *ΔcspAB* strains lacking detectable ActA displayed almost similar *actA* mRNA levels that were all significantly (*P<0.05*) higher than those of the WT strain, which contained high ActA protein amounts (Fig. 4).

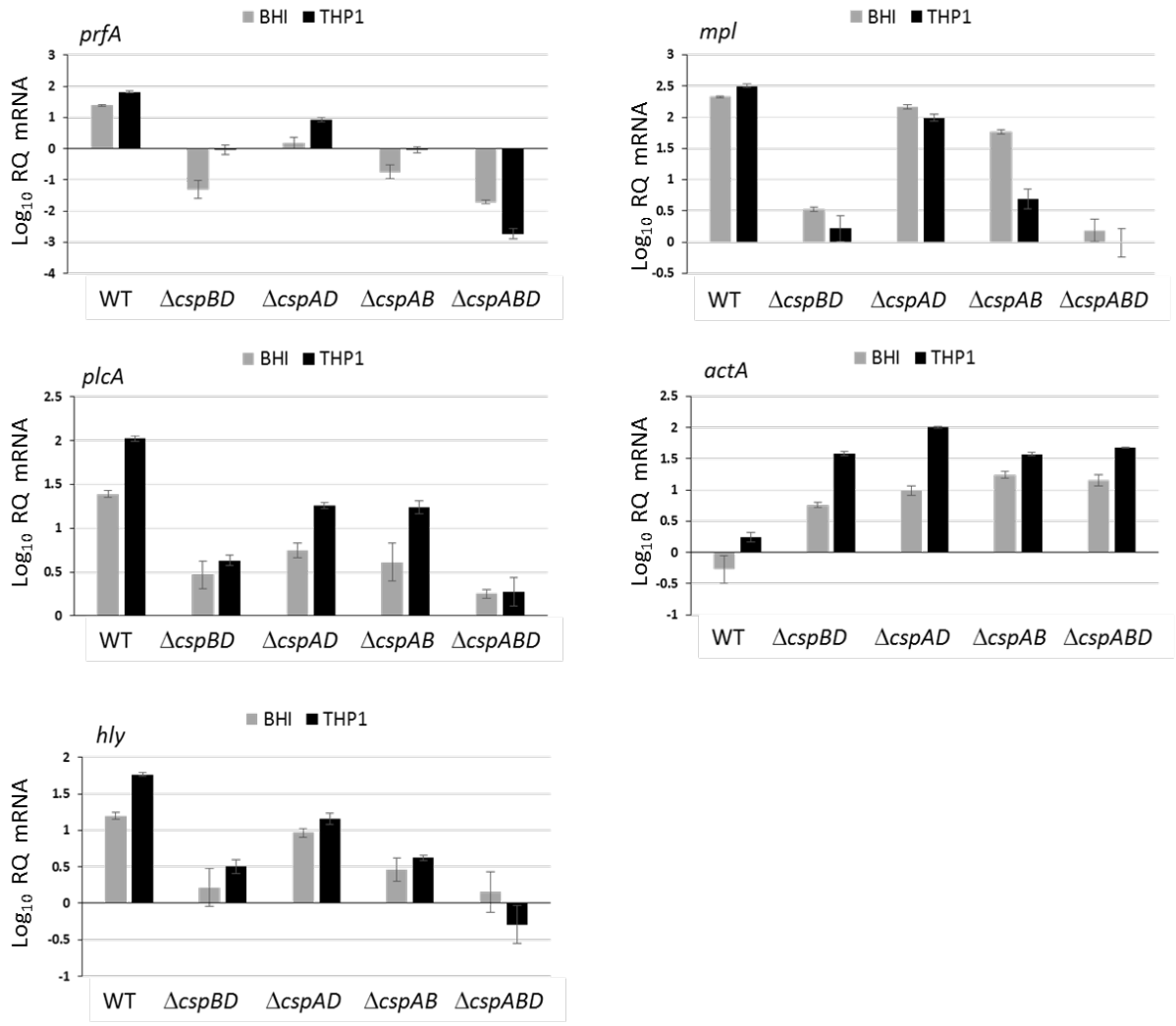


Figure 4. Impact of *csp* deletions on *prfA*, *hly*, *ActA*, *plcA*, and *mpl* mRNA levels. mRNAs in EGDe WT and *csp* mutants cultivated to stationary phase in BHI broth and those recovered six hours after infection of macrophages were quantified using real-time qRT-PCR. The mRNA levels are normalized to 16S rRNA and expressed relative to a calibrator mRNA sample based on BHI broth cultivated stationary phase culture of EGDe WT. Results

presented are the means (bars) and standard deviations (error bars) of three independent biological experiments.

Csps loss abrogates *L. monocytogenes* aggregation.

Besides roles in virulence the surface protein ActA is also required for *L. monocytogenes* aggregation. We thus hypothesized that Csps could also have an indirect impact on aggregation in this bacterium since they have an influence on ActA expression. Stationary phase EGDe WT and *csp* mutant strains cultivated in BHI were statically incubated at 37°C for 24 hrs. During this period the samples were monitored for aggregation based on visual, spectrophotometric and microscopic examination (Figs. 5A-C). Bacterial aggregation accompanied by decreasing optical density of the cultural supernatant was observed with the WT strain as well as with the CspB (*ΔcspAB*) producing strain but at a lower level. No significant aggregation occurred with the Csp devoid *ΔcspABD* as well as the CspA (*ΔcspBD*) and CspD (*ΔcspAB*) producing strains. The aggregation trends observed reflected ActA expression trends observed with the different *csp* mutants. Overall these results thus showed that Csps, particularly CspB, also indirectly contribute to ActA-dependent aggregation of *L. monocytogenes*.

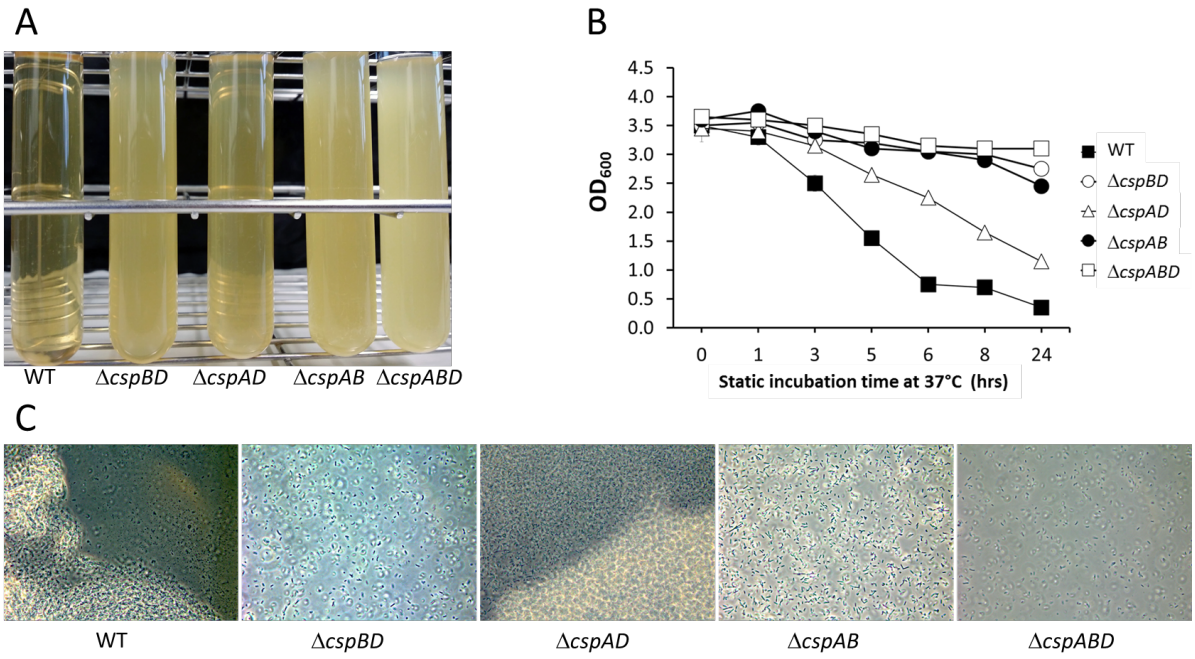


Figure 5. Impact of Csp loss on *L. monocytogenes* aggregation. The aggregation phenotypes of EGDe WT and *csp* mutants was assessed in stationary phase BHI cultures incubated under

static conditions for 24 h. (A) BHI culture sedimentation observed after 24 h, (B) Time resolved aggregation kinetics based decrease in OD_{590} of the supernatant of a static BHI culture as bacteria cells aggregate and sediments during 24 h, (C) Bright field microscopic examination of the sedimented bacteria after 24 h of static incubation.

L. monocytogenes csp mutants are impaired in swarming motility and flagella expression.

Swarming motility phenotypes between *csp* mutants and the WT *L. monocytogenes* EGDe were compared after growth on semi-solid BHI agar incubated for 48 h at 25°C (Fig. 6A). Csp devoid *ΔcspABD* and the CspD harboring *ΔcspAB* strain showed no motility, whereas the *ΔcspBD* and *ΔcspAD* strains harboring CspA and CspB, respectively, showed similar levels of swarming motility, which were significantly reduced compared to the parental WT strain. To further assess if the lack as well as reductions in swarming motility of the *csp* mutants was due to impaired flagella expression, the *csp* mutants and WT strain were examined for flagellation by electron microscopy. The motile WT strain showed peritrichous flagellation and both non-motile *csp* mutants (*ΔcspABD* and *ΔcspBD*) were non-flagellated (Fig. 7B). Both weakly motile mutants (*ΔcspAD* and *ΔcspAB*) showed presence of some flagellated cells (20-30%) that were characterized by a relatively fewer number of flagella per cell compared to the WT strain (Fig. 6B). Quantification of *flaA* mRNA transcripts encoding flagellin, the structural subunit of flagella by qRT-PCR showed that these transcripts were actually expressed at significantly higher levels ($P<0.05$) in all the *csp* mutants compared to the WT, including the non-flagellated and non-motile *ΔcspABD* and *ΔcspBD* strains (Fig 6C). Our observations thus showed that Csps are also functionally required for the expression of flagellation and extracellular motility of *L. monocytogenes*.

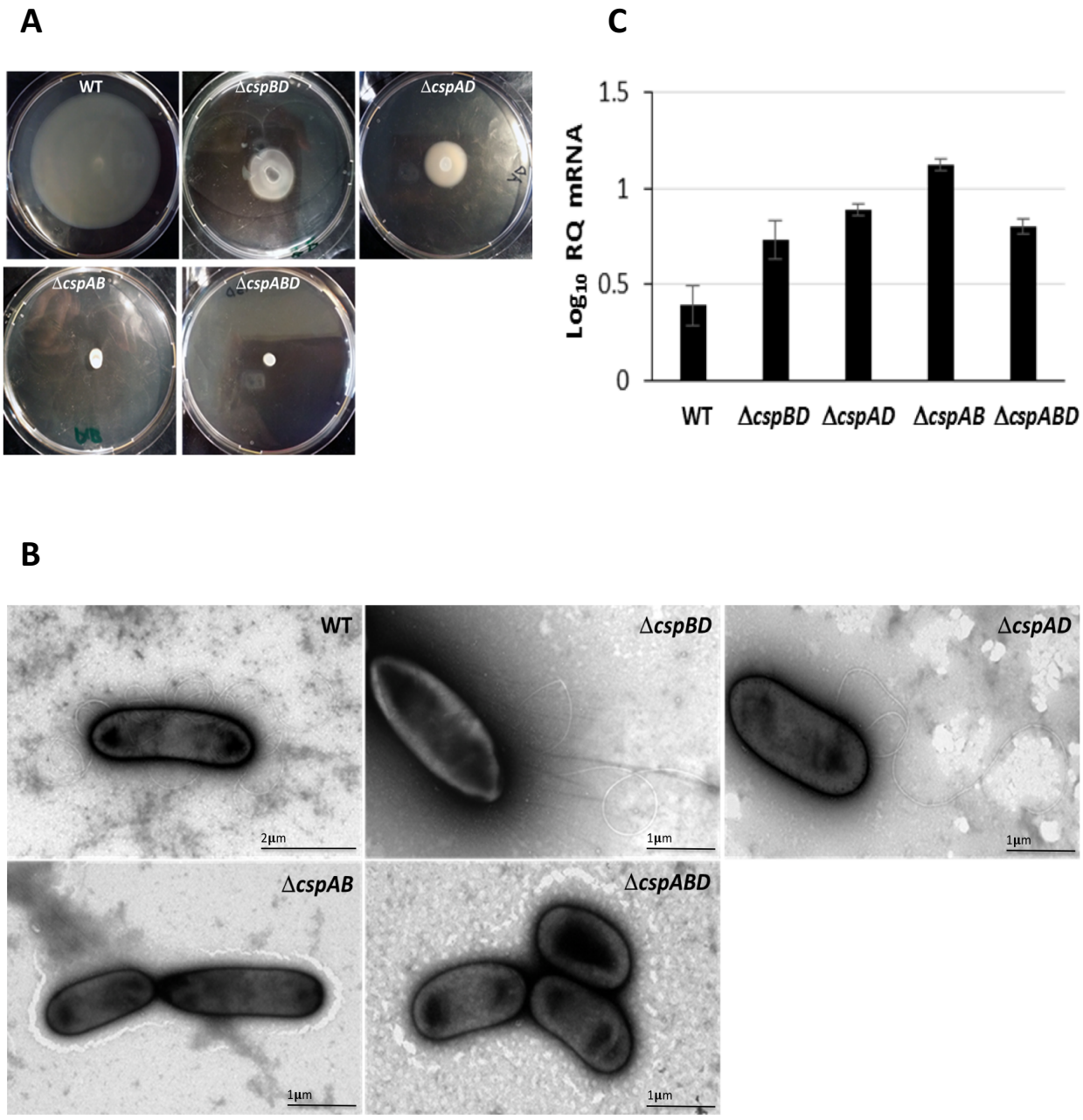


Figure 6. Impact of Csps on swarming motility, flagellation and *fla A* mRNA levels. (A) Swarming motility of EGDe WT and *csp* mutant strains spotted on 0.25% BHI agar and incubated at 25°C for 48 h, (B) Electron microscopic visualization of bacterium-associated flagella in EGDe WT and csp mutants (C) qRT-PCR quantification of *flaA* mRNA in EGDe WT and *csp* mutants cultivated to stationary phase in BHI at 25°C. The mRNA levels are normalized to 16S rRNA and expressed relative to a calibrator mRNA sample based on BHI broth cultivated stationary phase culture of EGDe WT. Results presented are the means (bars) and standard deviations (error bars) of three independent biological experiments.

Discussion

Csps-dependent regulation of gene expression is presumed important to various aspects of bacterial physiology including expression of virulence and stress resistance phenotypes that underlie current public health and food safety challenges posed by the foodborne pathogen *L. monocytogenes*. Previously we showed that besides promoting important stress resistance phenotypes such as cold and salt stress growth, Csps were also relevant for efficient human intestine epithelial cell invasion, as well as growth inside murine macrophages and LLO production in *L. monocytogenes* (30, 33-34).

The current studies were conducted in context of human macrophages as well as a zebrafish embryo based multicellular infection model revealing that Csp functions are necessary for the optimal virulence expression in this bacterium. In assessing growth and persistence inside human macrophages we found that *csp* mutants, which are devoid of all Csps (*ΔcspABD*) or produce single Csp variants are significantly attenuated compared to the parental WT strain of *L. monocytogenes* EGDe that harbors all the three Csps. Similarly, we also found that such mutants were attenuated in their capacity to induce pathology and mortality in zebrafish embryos relative to the parental WT strain.

A completely Csp devoid mutant *ΔcspABD*, showed the most attenuated phenotypes confirming the important functions served by Csp-dependent regulation in facilitating the expression of the examined virulence associated phenotypes in this bacterium. Using mutants producing single Csps, the roles of the individual Csp variants in these virulence phenotypes was assessed. This revealed that all the three Csps were functionally involved. Furthermore, it showed that the three *L. monocytogenes* Csp variants although having some functionally redundancy were not equivalent in roles regulating expression of mechanisms that facilitate survival and replication in human macrophages as well as virulence in zebrafish embryos. All three Csps expressed individually rescued *L. monocytogenes* growth and persistence in human macrophages and virulence in zebrafish embryo virulence relative to phenotypic levels of the Csp devoid (*ΔcspABD*) mutant. Overall the individual Csp enabled phenotypes showed a hierarchical trend of CspB>CspD>CspA in both infection models. None among the individual Csps could recapitulate phenotype levels exhibited by the WT strain

with all three Csps. This indicates that facilitation of maximal virulence expression depends on the production and co-operative function of all the three Csp variants although the main roles are provided through CspB.

Overall our analysis thus revealed that despite being highly conserved the three Csps display variability in their functional capacities to promote *L. monocytogenes* growth and persistence in human macrophages as well as zebrafish virulence. The mechanisms of such variability in Csp functions remain unknown although it could be due to differences in their expression regulation or their regulatory targets and nucleic acid binding modes and efficiencies.

In other bacteria Csp regulated genes have been shown to also include those encoding virulence associated proteins (27). Virulence gene expression regulation involves various protein based regulatory mechanisms in *L. monocytogenes* some of which are yet to be understood (37). Csps based on the nature of their proposed functions are expected to be among proteins involved in virulence gene regulating mechanisms of *L. monocytogenes*. Along these lines we previously showed that LLO expression is regulated through Csp-dependent mechanisms since LLO mRNA levels and stability are reduced without Csps (34). *L. monocytogenes* survival and replication in human macrophages and zebrafish virulence are mediated through virulence factors that include the proteins PrfA, LLO, Mpl, PlcA and ActA. Impairment of these virulence attributes in *csp* mutants appear to arise from the impaired expression of these virulence factors due to reduced (Δ*cspBD*, Δ*cspAD* and *ΔcspAB*) or complete (*ΔcspABD*) lack of Csp functions. All the *csp* mutants are characterized by significantly lower production of PrfA, LLO, Mpl, PlcA and ActA compared to the WT strain. With the exception of ActA, the low production of these proteins was associated with reduced amounts of their encoding transcripts in the *csp* mutants compared to the WT strain. As such it appears that the ability of Csps promote *L. monocytogenes* virulence, can in part be linked to their roles in facilitating expression of key virulence genes at mRNA and protein levels. Csp-dependent enhancement of mRNA levels might be associated with to the role of these proteins in transcription activation as well as mRNA stabilization (28, 38-39). Besides increasing transcript level, Csps could also enhance virulence protein synthesis through direct interaction with transcripts to enhance translation either by facilitating

translation initiation or through destabilization of translation inhibiting mRNA secondary structures (38-39). Meanwhile although *actA* mRNA levels that were significantly higher in the *csp* mutants, these mutants either lacked (*ΔcspABD* and *ΔcspAB*) or produce lower (Δ*cspBD* and Δ*cspAD*) ActA compared to the WT strain. It thus appears that *actA* transcripts in these mutants are not being efficiently translated to proteins due to Csp deficiencies. In addition to roles in facilitating intracellular motility and cell to cell dissemination, the surface protein ActA also mediates *L. monocytogenes* aggregation, which has been suggested to be an important attribute in long-term gut persistence to facilitate dissemination of this bacterium from infected hosts back to the environment (40). We show here that *L. monocytogenes* aggregation is also a Csp-dependent phenotype. Assessing for aggregation showed phenotypic trends that reflected ActA expression trends among the csp mutants. No aggregation was observed in *csp* mutants that lack (*ΔcspABD* and *ΔcspAB*) or expressed low (Δ*cspBD*) ActA amounts. Aggregation was observed with the CspB harboring Δ*cspAD* mutant but the level was significantly below WT strain levels. It thus appears, similar to what has been observed for PrfA, Csp function is also indirectly involved in promoting ActA-dependent aggregation in *L. monocytogenes* (40). Overall our observations suggest that Csp-dependent regulation of virulence gene expression probably involves both direct and indirect mechanisms. Direct roles being mediated through direct Csp targeting of *prfA*, *hly*, *mpl*, *plcA* and *actA* mRNAs at transcription and translation level. Csp-dependent regulation of PrfA expression allows indirect transcriptional regulation of these genes because PrfA is their master transcription regulator.

In other microrganisms such as *E. coli* and *B. melitensis* it has been reported that flagella genes are among genes regulated through Csps (27-41). Similar to such observations, we found here that the expression of flagella and flagella-based swarming motility is also Csp-dependent. Both flagellation and swarming motility were abrogated without Csps or when CspD only was produced. Meanwhile they were reduced upon CspB or CspA production. Overall it thus also appears that while CspD has limited roles, CspA and CspB are necessary for the optimal expression of flagella and swarming motility. Once again we also found that although impaired in flagella expression, the *csp* mutants also showed higher *flaA* mRNA encoding flagellin compared to the flagellated WT strain. An observation that further

suggest that translation is also impaired due to lack of Csp in these mutants. Meanwhile CspA and flagella are also both important for *L. monocytogenes* cold growth. Our data here suggest that one of the Csp-dependent mechanisms in *L. monocytogenes* cold growth promotion involves CspA roles in facilitating flagellation and swarming motility expression.

Overall our studies have now shown that in addition to previously ascribed roles in food-related stress protection, Csp functions are necessary for the maximal expression of different virulence associated phenotypes in *L. monocytogenes*. These proteins are important for *L. monocytogenes* virulence both in vivo and in vitro as demonstrated by the fact that a mutant of *L. monocytogenes* without Csps, is completely attenuated in survival and replication inside cultured human macrophages, and it fails to induce any detectable pathogenicity or mortality in zebrafish embryos. Furthermore, we show for the first time through this study that Csp functions are necessary in facilitating bacterial aggregation, flagella biosynthesis and flagella-based motility. Without Csps *L. monocytogenes* is unable to aggregate and fails to express flagella and exhibit swarming motility. Meanwhile Csps appear to regulate these phenotypes through mechanisms that involve transcription and translation level regulation of genes that encode for proteins, which are specifically involved in virulence (*prfA*, *hly*, *mpl*,*plcA* and *actA*), aggregation (*prfA* and *actA*) and flagella-based (*flaA*) motility functions. Csps of *L. monocytogenes* although highly conserved and sharing functionally redundancy they also clearly show variable functional relevance in regulating Csp associated phenotypes observed in this bacterium. Previously CspA has been found to be most important in cold adaptation and CspD in osmotic stress adaptation. In this study we show through analysis of here that CspB is the most relevant among the three Csps regarding regulation of genes related to virulence, aggregation and motility phenotypes.

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Chapter 7

General Discussion and Conclusion

The studies comprised in this work are focused on some of the key-research topics that need to be addressed in order to understand the pathogenesis of intracellular opportunistic pathogens. In **Chapter 2** we have addressed the possible influence of FkpA variants in intracellular survival of *Cronobacter* spp. in human macrophages and provided strong evidence that FkpA must be considered a virulence factor in *Cronobacter* spp., however due to amino acid variations its influence on macrophage survival and replication varies among strains and/or species. Further studies will be required to in order to explain possible correlation between FkpA protein sequence variation and macrophage survival ability in *Cronobacter* spp. Thus, the precise mechanism by which this protein provides protection from phagocytosis remains elusive. One may speculate that the chaperone function of this protein may stabilize certain functional determinants such as pores or efflux pumps, the impaired function of these may lead to a reduced capability to survive within macrophages. Further experimental investigations are planned to answer these questions.

In **Chapter 3** we have developed the first zebrafish infection model for *Cronobacter* spp. and investigated mechanisms of pathogenesis and the nature and action of putative virulence factors. Zebrafish as a model organism is very well established and have already been applied to study infectious diseases in vertebrates and has also been used as model for human medicine. We started with bath immersion technique to infect the zebrafish larvae with *Cronobacter turicensis,* a clinical strain responsible for the death of two neonates. Though we found bacteria to be present in the gut of the zebrafish larvae and other parts of the larvae, variations between single experiments made the bath immersion technique unreliable. Hence we decided to introduce the pathogen into the blood stream of the animal *via* microinjection technique. An injection dose of 10,000 CFUs into the cardinal vein of 2-day old zebrafish larvae did not result in any clinical signs of infection in the embryos and fluorescence microscopy showed that bacteria were cleared within 24 hours post injection. This finding correlates with studies done in human blood that *Cronobacter spp.* display only a ‘‘moderate’’ ability to survive in human blood or serum. Similar observations

were reported for zebrafish larvae infection studies with *Staphylococcus epidermidis* and suggested that yolk of zebrafish larvae could serve as an alternative injection site (Veneman et al., 2013).

Microinjection of as few as 50 CFUs of *Cronobacter turicensis* into the yolk sac of 2 dpf zebrafish larvae resulted in rapid multiplication of bacteria in the yolk sac. At 24hpi bacteria were disseminated into the blood circulation leading to severe bacteremia and finally death of the larvae within 72hpi. We observed by live imaging that macrophages and neutrophils were recruited to the infection site (yolk sac) but failed to stop replication and distribution of bacteria inside the yolk. We also observed that *C. turicensis* could multiply both extracellularly in the yolk and intracellularly in the macrophages and neutrophils. At 24 hpi, free bacteria as well as infected macrophages were lining up near the border of the yolk sac, where a traverse into the blood circulation and the surrounding tissue was observed. At later stages (30 hpi – 48 hpi), bacteria were found to be forming micro-colonies in small blood vessels, particularly those near to the eyes and brain which is a typical feature for septicemia and bacteremia in fish. Thus, during infection in zebrafish embryos, a combination of extracellular and intracellular replication of *Cronobacter* could be observed.
At the stages used here (2dpf), the blood-brain barrier is not yet fully formed in the zebrafish larvae. The maturation of blood-brain barrier occurs between 3dpf to 10dpf. In our future studies, it may be interesting to infect late larvae aged about 2 weeks which will have fully matured blood-brain barrier. Optical convenience and genetic manipulability will be not as good as the early larvae but might open up new ways for analysis of fully immunocompetent adaptive immunity to *Cronobacter* in zebrafish. Upcoming studies will focus on the elucidation of the molecular mechanisms involved in meningitis caused by *Cronobacter* spp. We are currently in the phase of adapting the zebrafish embryo model to study bacterial and host factors involved in crossing of the blood brain barrier which leads to the development of this clinical manifestation.

We further investigated whether antimicrobial compounds could be used to cure *Cronobacter* infection in our zebrafish model by application of the drugs to the fish water. Out of selection of antimicrobial compounds used nalidixic acid was the only drug that could

efficiently kill the bacteria inside that host. Although the survival rate of nalidixic acid cured infected fishes was close to 100 % after 72 hpi, pericardial edema was observed as a side effect. Interestingly, similar side effect has also been reported in humans such as cerebral edema and intracranial pressure after treatment with nalidixic acid (Rao 1974). Even though the antibiotic concentrations we used were previously shown to be lethal for the bacteria *in vitro*, antibiotics such as ampicillin and tetracycline proved to be less effective to treat *Cronobacter* infection in zebrafish model. These outcomes demonstrate the differences of drug effectiveness *in vitro* and *in vivo* and the need for drugs to be tested *in vivo* to study their efficacy and possible side effects.

In our recent *in vitro* study we have shown that *C. turicensis* can survive and replicate for up to 96 hpi in human THP-1 macrophages and FkpA protein is one of the main factor responsible for this ability. To confirm these findings *in vivo,* we infected zebrafish larvae with *C. turicensis* FkpA mutant and found that pathogenicity was strongly attenuated in zebrafish model. This finding provides proof that macrophages and other phagocytic cells can play a vital role in the process of traversing physical barriers such as epithelia and endothelia and by this promote further systemic dissemination of intracellular bacterial pathogens within the host organism.

With the experimental design established, results can be obtained within days, and the number of experiments can be easily scaled up. Knowledge gained from this study (chapter 3) provides a promising tool which will enable us to screen large numbers of bacterial mutants and strains for virulence-related factors of *Cronobacter* and to unearth more detailed features of *Cronobacter* virulence and the counteracting innate immune response in our upcoming studies.

In **Chapter 4** we investigated a diffusible signal factor (DSF)-type quorum sensing (QS) based regulatory system in *C. turicensis.* Previous studies in *Burkholderia cenocepacia* have shown to produce DSF-type quorum sensing signals to control biofilm formation and virulence (Bi et al, 2012). The RpfF/RpfR system was shown to be involved in sensing and responding to DSF signals. This signal/sensor gene pair is highly conserved in several bacterial species including *Cronobacter* spp. (Suppiger et al., 2013). However, in this study we could show for

the first time, that this signal/sensor regulatory system is functional in *C. turicensis* and influences typically quorum sensing regulated phenotypes as well as virulence through modulation of the intracellular c-di-GMP levels.
In order to obtain a more comprehensive picture of the features regulated by this system we are currently performing transcriptome analysis on wild type and mutant strains. The results will allow us to elucidate the regulatory pathway initiated by this system and compare it to the one described in *Burkholderia cenocepacia* and/ or other, more closely related species harbouring this regulatory system.

Apparently not all *Cronobacter* species are linked to infantile infections indicating possible variations in virulence among strains. Whole genome comparisons and *in silico* analysis have elucidated potential virulence factors, the presence/absence of which may explain the differential virulence behavior of strains. However, high-throughput validation of these virulence factors was not possible due to lack of appropriate neonatal animal model. In **Chapter 5** we have used our recently established zebrafish embryo model to determine the virulence spectrum displayed among species and strains within the *Cronobacter* genus. From our experiments we have shown that that presence of RepFIB-like "virulence plasmid" (Franco et al., 2011) strongly contributes to virulence. Furthermore, our results from the infection experiments have underpinned the importance of two putative virulence factors—the plasmid encoded *Cronobacter* plasminogen activator Cpa and chromosomally encoded Zpx in *in vivo* pathogenesis. With this study we could show that the zebrafish model can be used to identify virulence factors and perform *in vivo* infection studies on a large scale level which will boost the understanding on the virulence strategies employed by these pathogens.

Resistance to stress and expression of virulence are important physiological attributes of *L. monocytogenes* responsible for its associated public health and food safety problems. Several studies have linked small nucleic acid binding Cold shock domain protein (Csps) with various stress resistance phenotypes. However, their possible contributions to virulence and other phenotypes like motility still remains elusive. Recent studies in *S. aureus* and *Enterococcus fecalis* have linked Csps to the promotion of virulence related phenotypes. In **Chapter 6** we used the macrophage and the zebrafish infection model to study proteins of

the cold shock domain protein (Csps) family of another intracellular opportunistic pathogen, *Listeria monocytogenes.* In this study we have shown that Csps play an important role in survival and persistence of *L. monocytogenes* inside human macrophages. Furthermore, we have also provided evidence that these proteins are important in enabling the full expression of virulence of this bacterium within a zebrafish based infection model. Moreover, we also showed that Csps are functionally required for the facilitating bacterial aggregation, flagella biosynthesis and flagella-based motility. Although *csp* mutants showed higher mRNA levels of *actA* and *flaA* compared to wild type, they were not efficiently translated to proteins due to Csp deficiencies. In our future studies, it will be of significance to examine Csp-dependent enhancement of mRNA levels associated to the role of these proteins in transcription activation as well as mRNA stabilization. It would be of interest to see if Csps could also enhance virulence protein synthesis through direct interaction with transcripts to enhance translation by either facilitating translation initiation or through destabilization of translation inhibiting mRNA secondary structures. *L. monocytogenes* harbors three Csps (CspA, CspB and CspD). Analysis of such single Csp producing strains in human macrophage and zebrafish embryo revealed functional contribution of the individual Csps to *L. monocytogenes* virulence promotion. Finally, we have showed that expression of all PrfA regulated virulence genes is depressed/altered without Csps and impaired virulence was observed in human macrophages and zebrafish. It is well known that PrfA regulates *L. monocytogenes* virulence by switching on genes in the PrfA regulon.

By using two facultative intracellular pathogens we have demonstrated that zebrafish can be a promising and powerful model organism to identify virulence factors and to study and visualize host innate immune responses to bacterial infections in real time. Like any other animal model, zebrafish model also has few limitations such as they cannot be used to study infections by oral route (bath immersion) which would have been especially interesting when studying food associated pathogens. Furthermore, zebrafish studies are performed at 28°C (optimal growth temperature for zebrafish embryos) and it is known that some virulence genes are expressed at temperatures around 37 °C. Hence zebrafish infection model can only be seen as a screening system for virulence factors and to provide initial understanding of infection mechanisms. However, infection mechanisms will have to be reconfirmed with other (mammalian) models.

6.3 Conclusion

From our studies it is evident that the crucial virulence factor for facultative intracellular bacteria such as *Cronobacter* spp. and *Listeria monocytogenes* is its ability to survive and replicate inside macrophages and neutrophils. Further these phagocytes can be used by the bacteria to transverse various barriers and disseminate inside the host.

Our future work is to utilize transgenic and mutant strains of both zebrafish and bacteria to elucidate virulence factors of the pathogens and host receptors including the immune responses. Furthermore, we also plan to use RNA sequencing and microarray technologies to help determine virulence factors and host receptors in a high-throughput manner. The advantage to study the infection *in vivo* will also give the opportunity to address particular questions, for e.g. the crossing of the blood-brain barrier by pathogens such as *Cronobacter* and *Listeria*, a critical step in the etiology of meningitis.

The final results of this project will contribute to a better understanding of the pathogenesis of *Cronobacter and Listeria* infections and hopefully provide a basis for the development of new strategies to treat and/or prevent infections by these pathogens.

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